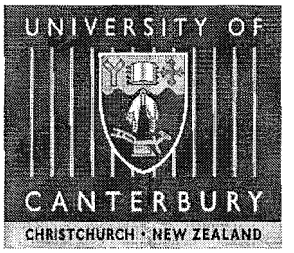
CIRCULATING CATECHOLAMINE CONCENTRATIONS AND EFFECTS ON THE CARDIOVASCULAR SYSTEM OF THE CHINOOK SALMON (*Oncorhynchus tshawytscha*) ON EXPOSURE TO COMMERCIALLY USED FISH ANAESTHETICS.

By Sarah Elizabeth Rothwell



A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Zoology

at the

University of Canterbury,

Christchurch, New Zealand

2004

Abstract

Whole animal, *in situ* and *in vitro* experiments were performed to investigate the dynamics of catecholamine release during anaesthesia and the consequent effects on the cardiovascular system of Chinook salmon. Blood pressure, heart rate, haematocrit, mean cell haemoglobin concentration (MCHC) and plasma catecholamine concentrations were used as indicators of cardiovascular function. *In vivo* experiments were performed to elucidate whether anaesthetic exposure *per se* stimulates catecholamine release. There was no correlation between changes in cardiovascular variables and plasma catecholamine concentrations during or after anaesthesia, suggesting that changes were due to anaesthetic induced hypoxia. When whole salmon blood was exposed to hypoxia, the pattern of Hct and MCHC changes were the same as those seen *in vivo*.

However, deep anaesthesia elicited strong catecholamine release in both Chinook salmon and snapper. Despite plasma catecholamine levels above 500nML^{-1} the circulating half-life in both species was <10 minutes. Changes in cardiovascular variables were consistent with typical adrenergic effects in teleost fish seen during hypoxia. These results suggest that anaesthetic induced hypoxia is the proximate stimulus for catecholamine release in anaesthetised salmon. However, *in situ* posterior cardinal vein (PCV) preparations did not secrete catecholamines when exposed to hypoxic saline, suggesting a higher control centre is responsible for hypoxic catecholamine release, *in vivo*.

AQUI-S anaesthetic had no direct effect on catecholamine secretion patterns in field stimulated PCV preparations. However, *in vitro* myography experiments and *in situ* perfused tail preparations indicated that both AQUI-S and MS222 are potent vasodilators of peripheral vessels in salmon.

Although anaesthesia *per se* does not stimulate catecholamine release in salmon, anaesthetic induced hypoxia does. Direct effects of anaesthetics on blood vessels may antagonise the actions of catecholamines released during hypoxia. This could have significant effects on cardiac return and gill perfusion patterns.

Table of Contents

Page No.

List of Figures.

List of Tables

Chapter 1

General Introduction.

New Zealand has a well-developed and internationally established commercial fishing industry. Worldwide, the demand for seafood is increasing to the extent that it is the fastest growing food sector in the world. Per annum, seafood sales increase at a rate of over 10% compared to other food sectors (GESAMP, 1997; NIWA website, 2003). Export revenue from the seafood industry accounted for over $170 million dollars of New Zealand's export revenue in 1999 with salmon product export accounting for 35% of the total (NIWA website, 2003; SeaFIC NZ web site, 2003). Three species of salmon have been successfully introduced into New Zealand, Chinook salmon (*Oncorhynchus tshawytscha*), Sockeye salmon (*Oncorhynchus nerka*) and Atlantic salmon (*Salmo salar*). Of these species, only Chinook salmon are successfully farmed. New Zealand's first commercial salmon farm was established in 1976 in Golden Bay and there are now 14 on-growing sites with 12 hatcheries and fresh water sites throughout the country (SeaFIC website, 2003). This represents approximately 60 hectares of farm, mostly found in the Marlborough sounds at the top of the South Island. All fresh water fisheries are found in the South Island and the existing capacity of the industry is about 10,000 tonnes with the ability to expand to 14,000 tonnes over the next decade (SeaFIC website, 2003). In 1997, New Zealand accounted for 9700 tonnes of worldwide Chinook salmon production (SeaFIC website, 2003). The aquaculture industry therefore, is one of the fastest growing sectors of the economy and has the potential to represent an increasingly significant proportion of New Zealand's export earnings.

Fish farming is similar to other livestock management with respect to maintenance of brood stock, hatcheries and quality of end product (Ross and Ross, 1984; Burka et al., 1997). The distinctive physiology of fish and their close contact with their environment present unique challenges for aquaculturists. Handling of fish both in and out of the water often creates great difficulties. Due to their intimate contact with the environment, fish are extremely sensitive to external stressors such as water pH, oxygen content and changes in temperature. Fish also respond violently to handling and their characteristic struggling during capture and handling has strong effects on both physiology and behaviour (Ross and Ross, 1984; Wendelaar Bonga, 1997). Physiological

stress upon removal from water can be compounded by the risk of serious abrasion and mechanical shock, especially with large groups of struggling animals (Ross and Ross, 1984). Even minor damage to the skin or gills can result in an imbalance in ionic regulation or reduction in blood oxygenation (Wendelaar Bonga, 1997). Mortality during transport is common due to increased metabolism (as a result of stress), which in turn can decrease water pH and PO_2 in closed systems (Brown, 1993; Burka et al., 1997).

Air exposure, crowding and chasing are well known to elicit intense stress responses from fish (Strange and Schreck, 1978; Laidley and Leatherland, 1988; Morales, et al., 1990; Sumpter et al., 1993; Reid, et al., 1994; Perry, et al., 1996; Wendelaar Bonga, 1997). Stressors have two fold actions; 1/ they produce effects that threaten or disturb internal homeostatic equilibrium and 2/ they elicit a set of behavioural and physiological responses that are compensatory and /or adaptive. However, intense chronic stress may decrease the adaptive value of the stress response, causing it to become dysfunctional (Wendelaar Bonga, 1997; Mommsen et al., 1999). Stress can lead to immediate or delayed mortalities, and often causes poor feeding reactions. This can inhibit gastrointestinal function, impairing growth, reproduction and reducing resistance to pathogens (Ross and Ross, 1984; Wendelaar Bonga, 1997; Mommsen et al., 1999; Perry and Bernier, 1999). Studies on the Antarctic charr (*Salvelinus alpinus*) show that subordinate fish have significantly lower specific growth rates and digestibility coefficients of food compared to dominant fish (Olsen and Ringo, 1999).

One of the primary stress hormones, cortisol, induces gluconeogenisis which involves deamination of amino acids from the skeletal muscle and increases blood glucose concentrations (van der Boon et al., 1991; Mommsen et al., 1999). Prolonged stress which raises plasma cortisol levels in the fish can lead to muscle atrophy, reduced feeding and inhibition of growth (van der Boon et al., 1991; Mommsen et al., 1999). Also formation of lactate in muscles due to struggling during stress may result in decreased meat quality of the final product (Burka et al., 1997). Additionally, metabolism of the white muscle of salmonids is influenced by catecholamines, which are the major stress hormones released during intense or chronic stress (Wendelaar Bonga, 1997; Perry and Bernier, 1999).

It is, therefore desirable to develop anaesthetic techniques that allow the transportation and handling of fish without impairing their health or commercial value (Bernier and Randall, 1998). Sedation during transport reduces metabolic rate and therefore oxygen consumption and also reduces excretion of metabolic products into the water (Ross and Ross, 1984; Woody et al., 2002). To try and reduce stress due to

handling, it has become common practice to anaesthetise fish prior to manipulation (Quinn et al., 1988; Ryan et al., 1993; Bernier and Randall, 1998; Woody et al., 2002). Anaesthetics are used for a number of fishery applications, ranging from mild sedation to prevent damage to fish during transport, grading, egg and milt stripping and vaccination (Ross and Ross, 1984; Brown, 1993; GESAMP, 1997; Bernier and Randall, 1998; Woody et al., 2002) through to general anaesthetization for marking, tagging and minor surgical procedures (Ross and Ross, 1984; Burka et al 1997). Over anaesthesia may also be used for harvesting large quantities of fish (AQUI-S website, 2003).

The second major group concerned with stress in fish are researchers. The effect of experimental procedure on the animal is a central question in physiology. Experimental procedures may have an important impact on the measurements of such parameters as metabolic rate, blood hormone concentrations and cardiovascular function (Randall, 1962). Factors such as handling and immobilization methods can have a considerable effect on the rate of oxygen consumption (Fry, 1957, cited in Randall, 1962; Walsh et al., 1998). Researchers primarily use anaesthetics in preparing fish for invasive procedures such as catheterisation or surgical ablation of glands for endocrine, pharmacological or physiological studies (Summerfelt and Smith, 1990). In these circumstances, general anaesthesia is necessary to minimise surgical stress. Mild sedation or immobilisation is usually required for procedures such as or field collection of animals or field studies of fragile or dangerous species or treatment of localised infections, fin clipping or measuring (Healey, 1964; Marking and Meyer, 1985; Woody et al., 2002).

Techniques of fish anaesthesia

There have been many techniques of anaesthesia described over the past 80 years ranging from such methods as addition of cocaine or brandy to the water immersing the fish through to physical methods such as a blow to the head (Healey, 1964). Other more modern methods include chilling fish with ice, gassing inhalant water with saturating levels of carbon dioxide (CO_2) gas (Bernier and Randall, 1998) or chemical anaesthesia (Brown, 1993). However, chilling may not induce a true anaesthetic effect (i.e lack of pain sensation) (Healey, 1964) and there are specific problems associated with the use of CO_2 anaesthesia that may reduce its efficacy compared to other anaesthetics (Gilderhus and Marking, 1987). Deep anaesthesia may not be achieved with CO_2 anaesthesia and stress hormones such as cortisol and catecholamines may be increased significantly with CO_2 anaesthesia (Gilderhus and Marking, 1987, Bernier and Randall, 1998). Blood lactate levels may also be increased following induction (Bernier and Randall, 1998) and the concentration of CO_2 in the immersing water is difficult to control (Brown, 1993).

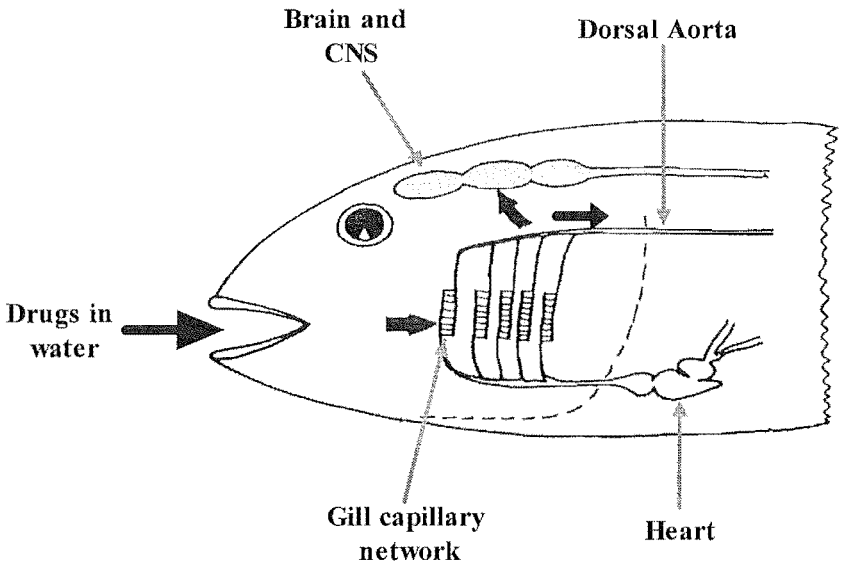


Figure 1.1 Diagram showing the route of absorption of inhalation anaesthetics in fish. The anaesthetic drug is dissolved in the water immersing the fish. The drug is then absorbed across the gill capillary network and travels in the blood to the brain and central nervous system (CNS). The drug is then distributed to the organs and other tissues via the dorsal aorta. (Taken from Ross and Ross, 1984)

The most popular alternatives to gas anaesthetics are chemical anaesthetics such as benzocaine, tricaine methane sulphonate (MS222), etomidate, and ketamine (Brown, 1993; Hill, 1999) Chemical anaesthetics may be administered to fish in several ways depending on the number of fish and levels of sedation required (Ross and Ross, 1984; Burka et al., 1997). Sedation methods include administration of anaesthetic agents in food pellets, or via intraperitoneal or intramuscular injections (Brown, 1993). These methods are obviously more suitable for anaesthetising either individual fish or very small numbers of fish. For large numbers of fish, the most popular choice of anaesthetic technique has been the use of 'inhalation' or 'immersion' anaesthetics (Burka et al., 1997, Brown, 1993; Bernier and Randall, 1998). These anaesthetics involve either adding the fish to a container of anaesthetic solution of desired concentration or the addition of a specific concentration of raw anaesthetic or volume of concentrated anaesthetic to the water in which the fish is held (Figure 1.1) (Hill, 1999). This allows rapid absorption of the anaesthetic across the respiratory epithelia of the gills and into the blood stream and central nervous system (CNS) (Ross and Ross, 1984; Brown, 1993).

Anaesthetics used in the present study:

A brief comparison between the properties of MS222 and AQUI-S is shown in Table 1.1.

MS222

The most well described inhalation anaesthetic is Tricaine Methane Sulphonate, or MS222, primarily because of its extensive use in research. It was accidentally discovered when looking for a synthetic substitute for cocaine (Brown, 1993) and is an isomer of benzocaine with the amine group in the meta position rather than the para position of the benzene ring (Alpharma, 2003). Additionally, MS222 has a sulphonate radical making it more soluble but also more acidic in solution (Brown, 1993; Alpharma, 2003). Therefore, when used in solution, MS222 is usually buffered with bicarbonate. Physically, MS222 is a provided as a fine, white, crystalline powder that is highly soluble in water (solubility 1g/0.8mL) (Hill, 1999). MS222 is also highly lipid soluble. There is often an interaction between anaesthetic concentration and the age and weight of fish (Ross and Ross, 1994).

Known since about 1920, MS222 is classed as a local anaesthetic in mammals but acts systemically in fish by blocking neuronal sodium (Na^+) channels, thereby reducing transmission of nerve action potentials (Burka et al., 1997). MS222 is rapidly absorbed by the gills and has direct actions on the CNS, cardiovascular system, neuromuscular junctions and ganglionic synapses (Randall, 1962; Pierce and Pierce, 1967; Fromm et al., 1971; Lochowitz et al., 1974; Frazier and Narahashi, 1975; Soivio et al., 1977; Ross and Ross 1984; Brown, 1994; Ryan, 1993; Hill, 1999). MS222 is the most widely used anaesthetic in aquaculture practice today, and its popularity is probably due to its relatively low cost and ease of use compared to other anaesthetics (Ross and Ross, 1984; Alpharma, 2003). MS222 is the only chemical anaesthetic agent currently registered with the United States Food and Drug Administration (FDA) for use with 'food fish'. However, MS222 has a withdrawal period of 21 days prior to human consumption and therefore it cannot be used for harvesting meaning other potentially stressful methods must be used (e.g CO_2 gassing).

Due to its popularity in both aquaculture and research, the physiological effects of MS222 have been extensively studied in many fish species such as the dogfish, *Squalus acanthias* (Pierce and Pierce, 1967) the tench, *Tinca tinca* (Randall, 1962) the snapper, *Pagrus auratus* (Ryan, 1991) the rainbow trout, *Oncorhynchus mykiss* and *Salmo gairdneri* (Fromm et al., 1971; Lochowitz et al., 1974; Soivio et al., 1974a; Soivio et al.,

1977; Soivio and Hughes, 1978; Ryan et al., 1993) the Chinook salmon, *Oncorhynchus tshawytscha* (Hill, 1999) and squid (Frazier and Narahashi, 1975).

AQUI-S

AQUI-S was first approved for use with fish and other seafood in 1994. It is the only anaesthetic designed specifically for use with fish and was developed by the Seafood division of Crop and Food, a New Zealand Crown Research Institute (Hill, 1999; AQUI-S website, 2003). Consisting of roughly 50% isoeugenol (a major component of clove oil) and 50% of a commercial food-emulsifying agent, AQUI-S is a thick viscous golden coloured liquid, which smells of and appears very similar to liquid honey. Although soluble in water, it requires good mixing and when added to the inhalant water in high concentrations, fish often appear to be distressed, responding with strong swimming movements for several seconds (Hill, 1999). High concentration stock solutions (e.g above 100ppm) of AQUI-S are unstable in solution after approximately 8 hours and lose their anaesthetic properties (pers.obs). It has been reported as a biodegradable 'stressless' anaesthetic that inhibits dramatic increases in tissue lactate levels (Burka et al., 1997). Most fishery applications require a concentration of between 15 and 20mgL^{-1} and typical application costs for farmed salmon are 2-3 cents (US)/kg fish harvested (AQUI-S website, 2003). A major advantage of AQUI-S is the fact it is currently approved in New Zealand, Australia and Chile for use with seafood with a nil withholding period. As such, it has been used in New Zealand for the harvesting of salmon since 1994 and is also widely used for transportation of lobster, eel and other fin fish (AQUI-S.com, 2003).

Studies on fish physiology specifically using AQUI-S anaesthetic are limited. However, those studies addressing the use of AQUI-S have focussed on efficacy (Iversen et al., 2003), tissue accumulation (Kildea et al., 2004) and effects on cortisol secretion (Mills 1998; Davidson et al., 2000; Iversen 2003). It is useful therefore to consider studies investigating the primary component of AQUI-S, clove oil. Clove oil is a distillate of the herbaceous parts of the clove tree, *Eugenia aromatica*, and contains approximately 70-90% eugenol (or isoeugenol), >17% eugenol acetate and up to 5% of Kariofilen 5 (Soto and Burhanuddin, 1995; Woody et al., 2002). Clove oil is considered non-mutagenic and safe by the U.S Food and Drug administration (Woody et al., 2002). The efficacy of clove oil has been investigated using several species such as the rabbit fish, *Siganus lineatus*, (Soto and Burhanuddin, 1995) the rainbow trout, *Oncorhynchus mykiss*, (Anderson et al., 1997; Taylor and Roberts, 1999) and white sturgeon (Taylor and Roberts, 1999). Clove oil has been shown to induce surgical depth anaesthesia in adult rainbow trout at

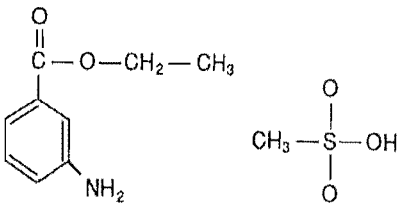
concentrations of 30mgL^{-1} (Prince and Powell, 2000) although mean lethal concentrations vary widely between species i.e 62mgL^{-1} for Chinook salmon versus 526mgL^{-1} for white sturgeon (Taylor and Roberts, 1999). Although clove oil has the advantage of inducing anaesthesia rapidly when compared to MS222, recovery times of up to 10 times longer than equivalent MS222 doses have been reported (Keene et al., 1998; Woody et al., 2002). The recommended dosage of AQUI-S for fishery applications is 17ppm (AQUI-S product information sheet). Recent studies have shown that at this concentration, AQUI-S causes prolonged elevation of cortisol levels in both chinook salmon (Mills, 1998) and immature rainbow trout (Davidson et al., 2000) following 30-minute exposure. However, Iversen et al., (2003) found that AQUI-S solutions ≥ 20ppm prevented plasma cortisol elevation in fresh water atlantic salmon (*Salmo salar*). In the channel catfish (*Ictalurus punctatus*) high concentrations of clove oil (200ppm) have also been shown to prevent cortisol elevation following a 30-minute exposure (Small, 2003). It is important to remember that AQUI-S is not directly analogous to clove oil. Clove oil does not fully dissolve in water at temperatures below 15°C (Woody et al., 2002). AQUI-S however does dissolve below this temperature (personal observation). There are invariably other differences between the 2 anaesthetics but these are not well described given the relatively unknown physiological effects of both anaesthetics.

Anaesthetic effects on the Cardiovascular system of Fishes.

Although fish anaesthetics are widely used, specific investigation into their effects on the cardiovascular system of fish have been relatively limited. Most literature concerning anaesthetic use has focused on cortisol induced stress responses (Hill, 1999). Many of these studies present conflicting data in terms of the biochemical and cardiovascular variables observed (Black 1964 cited in Randall et al., 1965) or they use spinally transected fish in order to reduce 'noise' on electrocardiograms used to monitor heart activity (Hill, 1999). Using this technique however, it is unlikely that responses observed in the animal are indicative of a natural response in the 'whole' animal.

Randall and Smith (1967) investigated the effects of different experimental procedures on the respiration and circulation of trout, tench, goldfish and salmon. They concluded that experimental technique had a large effect on these variables, and that wherever possible experiments should be carried out on unrestrained, intact, unanaesthetised fish. Clearly these criteria are difficult to meet. Monitoring of heart rate is

MS222



Active Component:
3-Aminobenzoic acid ethyl ester.

Common Name(s):
TMS
Finquel
TS-222 Sandoz
Tricaine-Sandoz

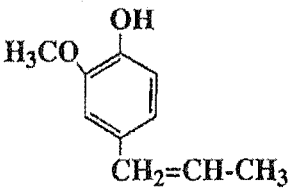
Solubility in Water:
Highly soluble but forms acidic solutions.
Requires buffering e.g with Na_2HCO_3

FDA Rating:
Only chemical anaesthetic currently approved for use with food fish but has a 21 day withdrawal period

Background and Uses:
Known since ~1920
Discovered when looking for an alternative to cocaine anaesthesia
Is a derivative of Benzocaine
Used internationally for fishery applications such as:
Spawn taking
Tagging
Fin Clipping
Transportation
Research applications

Contra-indications/Precautions:
Can cause hypoglycaemia in some species
Can be toxic to fish in saltwater
Has a limited safety margin; doses above 100ppm can be lethal
Has obvious physiological effects

AQUI-S



Active Component:
Isoeugenol

Common Name(s):
AQUI-S™
AQUI-S II

Solubility in Water:
High solubility, but requires through mixing
Anti-foaming agent required when used in water with air stones/pumps

FDA Rating:
Application for approval has been submitted

Background and Uses:
Similar to Clove oil anaesthetic.
Developed in New Zealand in the early 1990's specifically for use with food fish.
Used in New Zealand since 1994 and now Australia for 'rested harvesting' of salmonids and transportation of other seafood species.
Used in Norway for sedation of fish during vaccination

Contra-indications/Precautions:
May cause some initial irritation to fish when used at concentrations higher than >50ppm
May have significantly longer recovery times compared to MS222

Table 1.1 Table showing comparisons between the two commercial anaesthetics, MS222 and AQUI-S.

possible in unrestrained fish, but blood pressure or any other cardiovascular variables are impossible to measure without some type of surgery, restraint, or sedation of the fish. The aim is to separate effects caused by technique from those due to natural functions.

The catecholamine stress response.

To understand the effects of anaesthesia on fish physiology, it is a necessity to understand those elements involved in a typical response to stressors. Stressors produce effects that threaten or disturb internal homeostatic equilibrium. Physiological responses to a stressor are either specific for a single stressor or a group of related stressors or non-specific (Wendelaar Bonga, 1997). Although there are many different hormones involved in a co-ordinated stress response, catecholamines such as adrenaline and noradrenaline play one of the most dominant roles (Wendelaar Bonga, 1997)

Catecholamines include adrenaline, noradrenaline and dopamine. They are synthesized in the non-neural chromaffin cells and adrenergic neurons, via a series of enzymatic reactions. Chromaffin cells are areas of tissue associated with the head kidney of the teleost (bony) fish. Circulating catecholamine levels have many significant physiological effects, both direct and indirect. These effects can lead to an increase in or maintenance of energy turnover and oxygen supply under adverse conditions such as hypoxia or acidosis (Randall and Perry, 1992). Hypercapnia (increased CO_2) and hypoxia can depress glycogen synthesis and increase glycogenolysis (Randall and Perry, 1992) A rise in circulating catecholamines stimulates glycogen breakdown and maintains glucose availability (Wood et al., 1990 cited in Randall and Perry 1992). Catecholamines also increase ATP turnover in trout red blood cells during acidotic conditions, e.g. exhaustive exercise (Walsh, 1998) and play a major role in controlling oxygen delivery to tissues in teleosts by modulating changes in gill diffusing capacity, increases in erythrocyte number, volume and intracellular pH and redirecting blood flow (Randall and Perry, 1992). In many, but not all species of fish, the most significant effect of catecholamines is to enhance haemoglobin affinity for oxygen during hypoxia. This is achieved by elevation of erythrocyte intracellular pH by stimulation of Na^+/H^+ exchange across the cell membrane causing the phenomenon known as red blood cell swelling.

Hypoxia also causes red blood cells to swell. In response to changes in plasma pH and Hb-O_2 saturation, there are alterations in the Donnan distribution ratios of osmotically active ions and therefore water (Gilmour, 1998). In teleost fish, the erythrocytes are more permeable to small anions such as Cl^- and HCO_3^- than Na^+ or K^+ (Nikinmaa and Salama,

1998). Therefore, changes in haemoglobin charge are mainly compensated for by a net efflux or influx of Cl^- (Nikinmaa and Salama, 1998). A decrease in oxygen tension increases red blood cell (rbc) volume because as haemoglobin binds protons on deoxygenation, its negative charge decreases and Cl^- enters the cell (Nikinmaa and Salama, 1998). Osmotically obliged water follows and the cell swells (Nikinmaa and Salama, 1998; Gilmour, 1998; Gibson et al., 2000).

Red blood cells can also swell via a beta-adrenergically stimulated Na^+/H^+ antiporter in the cell membrane. Stimulated by a rise in the intracellular cAMP concentration (Nikinmaa, 1990; 2001) protons are pumped out of the cell in exchange for sodium ions and again, water follows Na^+ and Cl^- osmotically, causing the cell to swell. However, in this case because protons are being pumped out of the cell, the plasma pH drops. Maintenance of erythrocyte pH during acidosis protects against the Root shift (Randall and Perry 1992) allowing continued delivery of oxygen to tissues under adverse conditions and also maintaining CO_2 excretion. This is an adaptive response to hypoxia, as teleost blood has a marked 'Root' shift i.e. a reduction in blood pH leads to a decrease in oxygen binding capacity. The Root effect can be brought about by CO_2 accumulation when ventilation is depressed. Unlike mammals, fish capillary endothelium has no role in CO_2 excretion. Instead, all CO_2 transfer occurs through the red blood cell with a tight coupling of oxygen uptake and CO_2 excretion (Randall and Perry 1992). This coupling means that protons released during haemoglobin oxygenation supply protons for bicarbonate dehydration and therefore CO_2 excretion (Gilmour, 1998).

α-adrenergic stimulation of the spleen by catecholamines induces the release of stored red blood cells from the organ during hypoxia and following exhaustive exercise. This response is dose dependant in hypercapnic trout and is the dominant response leading to an increase in arterial blood oxygen content (Nikinmaa and Salama 1998). Catecholamines are also involved in regulation of the heartbeat and stroke volume. Holmgren, (1977) investigated the possibility of cardiac regulation by circulating catecholamines in the Atlantic cod, *Gadus morhua*. This study identified the presence of excitory adrenergic innervation acting on β-adrenoreceptors in the cod heart, and suggested that catecholamine release from chromaffin tissue in the head kidney may have an important regulatory capacity. Excitory adrenergic innervation has also been demonstrated in the heart of the marine teleost *Girella tricuspidata* (Montgomery et al., 1986). Interestingly, Montgomery et al., abandoned the use of MS222 as an anaesthetic for experiments due to inconsistent results most likely due to autonomic suppression. In most teleost fish, exercise causes an increase in adrenergic tone to the heart and a rise in

blood pressure is modulated neurally via α-adrenergic vasoconstriction in the systemic circulation, but a vasodilation in the branchial circuit (Randall and Perry 1992).

Catecholamine release.

The primary mechanism leading to catecholamine secretion from chromaffin cells of the teleost head kidney is believed to be increased neuronal stimulation by pre-ganglionic nerve fibres. When stimulated, the release of acetylcholine (a neurotransmitter) stimulates cholinergic receptors, ultimately initiating a series of Ca^{2+} dependant events ending in catecholamine secretion (Randall and Perry, 1992; Wendelaar Bonga, 1997; Reid et al., 1998; Perry and Bernier, 1999). In teleost fish such as the salmonid family, it is generally accepted that nicotinic receptors are the dominant cholinoceptor present on chromaffin cells (Reid et al., 1998). The nicotinic nature of cholinergic induced catecholamine secretion in teleost fish has been proven in several studies. The Atlantic cod, *Gadus morhua* releases catecholamines in *in situ* preparations in response to either electrical stimulation of the sympathetic nerves or application of acetylcholine (Perry and Bernier, 1999). This secretion can be inhibited or stopped by the addition of the ganglionic blocker hexamethonium, which inhibits nicotinic cholinoceptors (Reid et al., 1998).

However, nicotinic receptors are not the only receptor type found on chromaffin cells. Across vertebrate families, muscarinic receptors are found to be involved in stimulation of catecholamine secretion. Indeed they appear to have many different functions. In many non-piscine vertebrates, stimulation of chromaffin cells with muscarinic agonists has various effects including both enhancing and inhibiting nicotinic evoked release, stimulating catecholamine secretion in the absence of nicotinic agonists or having no effect at all (Reid et al., 1998). Recently, there has been some evidence to suggest that muscarinic receptors on the chromaffin cells of fish may also have various effects on catecholamine secretion. Montpetit and Perry, (1999) used a perfused posterior cardinal vein (PCV) preparation to determine the relative importance of nicotinic and muscarinic receptors in catecholamine secretion in the rainbow trout, *Oncorhynchus mykiss*. Using various nicotinic and muscarinic agonists and antagonists it was found that muscarinic cholinergic stimulation enhanced nicotinic evoked secretion. Of note in this study was the result that noradrenaline and adrenaline secretion did not always follow the same pattern. When nicotine and oxotremorine (a muscarinic agonist) were administered together, adrenaline secretion displayed a simple additive effect whereas the noradrenaline secretion nearly tripled under the same conditions (Montpetit and Perry, 1999; Lapner et al., 2000). When these drugs were applied individually to the preparation,

they had very little influence on noradrenaline secretion. Also oxotremorine administration caused a sustained release of both catecholamines while nicotine-evoked catecholamine secretion occurred transiently.

Work by Monpetit and Perry, (1999) has been further supported by a recent study again on the rainbow trout *Oncorhynchus mykiss*. Lapner et al., (2000) demonstrated both in chronically cannulated fish and PCV perfusion preparations that desensitisation of the chromaffin cell nicotinic receptors in trout does not inhibit catecholamine secretion during acute hypoxia. The ability of muscarinic or other agonists stimulating catecholamine release may become important during times when the predominant (nicotinic) pathway is impeded, particularly during stress.

Aims of this study

The above actions of catecholamines briefly summarize their importance in teleost fish in terms of homeostatic maintenance in the face of external stressors. If these responses are 'blocked' or 'dampened' by drugs such as those used as fish anaesthetics, then it is important to understand the physiological consequences. The aim of this research was to determine whether there was a significant increase in plasma catecholamine secretion during different levels of anaesthesia and whether increasing depths of anaesthesia modulated the normal actions of catecholamines *in vivo*. A further aim was to elucidate whether anaesthesia itself, or anaesthetic induced hypoxia is the proximate stimulus of catecholamine release from the chromaffin tissue.

Chapter 2

Cardiovascular and haematological responses of Chinook salmon and Snapper during anaesthetic induction and recovery.

Introduction

As previously described, fish can release catecholamines in response to a variety of stressors that generally require either a modification of cardiorespiratory function, or mobilization of energy sources (Reid et al., 1998; Perry and Bernier, 1999). These stressors can range from exhaustive exercise to anaemia to acid infusion (Reid et al., 1998). Historically, anaesthesia during either experimental or commercial manipulation has widely been accepted as a strong potentiator of catecholamine release in fish, particularly salmonids (Randall and Perry, 1992; Wendelaar Bonga, 1997; Reid et al., 1998; Perry and Bernier 1999). More recently however, it has become apparent that catecholamine release in fish varies greatly between species and between stressors. Indeed, several investigations have indicated that catecholamine release during stress is most likely due to hypoxemia rather than blood acidosis or direct anaesthetic effects (Aota et al., 1990; Iwama et al., 1989; Perry and Bernier, 1999) and in salmonids occurs at a critical PaO_2 value corresponding to the P_{50} (50% (Hb)-O_2 saturation) (Fiévet et al., 1990; Randall and Perry, 1992; Reid et al., 1998)

The majority of investigations into catecholamine release during hypoxia have focussed almost exclusively on trout (*Oncorhynchus mykiss)* and have monitored catecholamine effects on ventilation, blood pressure, heart rate and oxygen transport after short periods of hypoxia (Holeton and Randall, 1967; Tetens and Lykkeboe, 1988; Fritsche, 1990; Perry et al., 1996; Perry and Gilmour, 1996).

The clearance rate and specific half-life of adrenaline and noradrenaline in teleost fish have been given little attention and have again been mostly limited to the trout

Oncorhynchus mykiss and cod, *Gadus morhua* (Mazeaud and Mazeaud, 1973, Ungell and Nilsson, 1979; Nekvasil and Olson, 1986). Given that the effects of catecholamines produced during stress in fish can be very long lived, it is surprising that more studies have not examined cardiovascular and heamatological changes over several hours following an hypoxic event.

During investigation of the cardiovascular and respiratory effects of several anaesthetic agents on Chinook salmon (*Oncorhynchus tshawytscha)* Hill, (1999) found that during a 5-minute anaesthetic induction with AQUI-S, plasma adrenaline and noradrenaline levels rose when accompanied by a fall in PaO_2. However, those fish treated with either Metomidate or MS222 showed no elevation in plasma catecholamine levels despite a fall in PaO_2. Construction of a blood oxygen equilibrium curve indicated that those fish that did release catecholamines did so at a PaO_2 value below the P_{50} of the blood. However, not all blood samples with a PaO_2 less than the P_{50} contained elevated catecholamine levels (Hill, 1999)

Hill's research suggests that anaesthesia *per se* does not necessarily induce catecholamine release in the Chinook salmon. The purpose of this study therefore, was to extend this research and measure circulating catecholamine levels in Chinook salmon (*Oncorhynchus tshawytscha) in vivo* before, during and after induction of anaesthesia. Measurement of cardiovascular variables such as heart rate and dorsal aortic blood pressure as well as mean cell haemoglobin concentration and haematocrit allowed the effects of anaesthesia and/or catecholamines on the cardiovascular physiology of the whole animal to be monitored.

Methods and Materials

Series 2.1. Cardiovascular changes and haematology of Chinook salmon during and after anaesthesia.

The Chinook salmon (*Oncorhynchus tshawytscha)* used in Series 2.1 were donated from Issac's Salmon Farm, McLean's Island, Christchurch. After transport to the University of Canterbury in chilled, oxygenated water they were stored in a large (1.5 x 1.5 x 1.5 m), outdoor tank with constantly flowing fresh artesian water. The tank water came from the same source as that used in the experiments and had a mean temperature of 12.5 ± 2.5°C.

Experiment 2.1. Measurement of cardiovascular and blood variables of Chinook salmon during recovery from anaesthesia.

When required, Chinook salmon (1047.0 ± 227.0g, mean ± 1SEM, range 844 - 1562g, n = 7) were removed from the holding tank with a net and placed in a bucket of MS222 (100ppm MS222 neutralised with 300ppm $NaHCO_3$) until surgical anaesthesia was reached (gill ventilation had stopped). Fish were then placed ventral side up on an operating sling and a piece of cannula (1 mm OD, 0.5mm ID, approximately 1 m long) was inserted into the dorsal aorta via the roof of the mouth. A small puncture was made in the tough skin along the midline of the mouth, between the second gill arches, using an 18-gauge hypodermic needle. An internal trochar (sharpened 0.019 silvered steel guitar wire, Darco strings) within the cannula was pushed through the hole at a 45° angle until the tip of the trochar touched the spine. The trochar was then withdrawn, pulling blood up the cannula. The cannula was moved 10cm into the dorsal aorta and one ml of heparinized saline solution was slowly injected into the blood vessel. The cannula was exteriorised through the snout via a larger diameter cannula (1.98 mm OD, 1.02 mm ID, two cm long). The gills were flushed with aerated MS222 (50ppm MS222 + 150ppm $NaHCO_3$) periodically throughout the procedure. The fish were placed in clear perspex experimental containers (0.95 x 0.15 x 0.15 m) (see Figure 2.1 for experimental set up) with a fresh running water supply at 12.5°C for 48 hours to recover. The containers were covered with black polyethene to shield the fish from any external stimuli that may have disturbed them.

Several cardiovascular variables were monitored before and after induction. Dorsal aortic blood pressure (DAP, cm H_2O) and heart rate (beats per minute, bpm) were measured by connecting the cannula to a disposable pressure transducer (PVB 6003, Surgicare LTD, Victoria, Australia) linked to a digital amplifier (Power Lab 400 Quad bridge, ADInstruments, Dunedin). This allowed both graphic and digital data collection on a computer using appropriate software (Power Lab 'Chart', version 3.4 ADInstruments). The cannula was also used to extract whole blood samples (300 µl) allowing determination of haematocrit (%), mean cell haemoglobin concentration ($g.dL^{-1}$) and plasma catecholamine concentration (nM). After 48 hours the fish were exposed to a 5-minute anaesthetic induction with 60ppm AQUI-S. The anaesthetic solution was introduced via the same inflow valve as the normal water supply (Figure 2.1). Snap

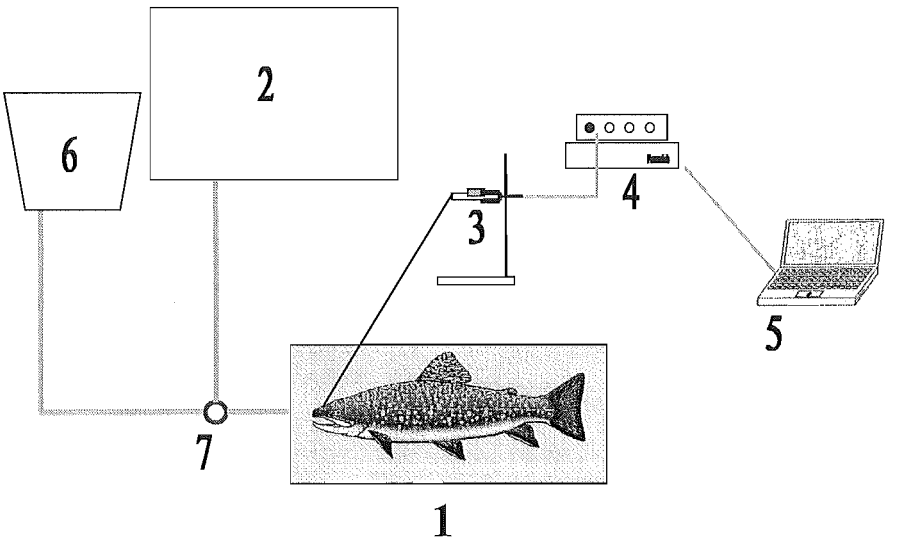


Figure 2.1. Diagram of experimental set-up for series 2.1 experiments. After surgery, fish were recovered in experimental boxes **(1)** for 48 hours prior to experimentation. The box was supplied with constantly fresh flowing tap water **(2)**. The aortic cannula was connected to a pressure transducer **(3)**. Blood pressure data was digitized using a Powerlab data acquisition system **(4)** linked to a laptop **(5)** which displayed the data graphically and calculated instantaneous heart rate. When required, anaesthetic solution from a bucket **(6)** could be exchanged for the fresh water supply via a 2-way tap **(7)**.

connectors (Nylex) on the hose from the anaesthetic bucket and the experimental tank, allowed rapid connection to the valve with minimal disturbance to the fish. Prior to induction, dorsal aortic blood pressure and heart rate were recorded for two minutes as a control, and were then continuously recorded throughout the induction period. A resting blood sample was also taken. Immediately after the induction period, a second blood sample was taken as above, and blood pressure and heart rate recorded. Blood samples, dorsal aortic pressure and heart rate were recorded at intervals over the following 6 hours post-induction.

For haemoglobin analysis, 10μl of blood was diluted 100 fold with Drabkins solution and stored at 4°C until required. Samples were analysed by measuring light adsorption at λ=540 nm against a haemoglobin standard (SIGMA kit, Sigma Chemical Company, St Louis) using a spectrophotometer (UV Kontron Spectrophotometer 860). Haematocrit was determined by spinning 60μl whole blood in heparinized microcapillary tubes (Clay Adams, New Jersey) in a Heraeus Haemofuge at 5000g for 5-minutes. The remaining blood was spun and the plasma collected and stored with EDTA/reduced

glutathione (5µl, 0.2M / 0.2M) at -70°C for less than 2 months prior to catecholamine analysis.

Plasma catecholamine samples were analysed using high performance liquid chromatography. To extract the catecholamines, 105 µl of plasma was added to 200 µl TRIS/EDTA (1M / 50mM) and about 10 mg of alumina. This was mixed thoroughly for 15 minutes, allowing the catecholamines to be adsorbed onto the alumina. The samples were then spun for 1 minute, the supernatant removed and the pellet washed for 5 minutes with 500 µl distilled water. This process was repeated twice to give a total of three distilled water washes. Finally, the catecholamines were eluted off the alumina by mixing with 150 µl acetic acid (0.2 M) and spinning for two minutes. The supernatant was injected into the column of a high performance liquid chromatograph (HPLC, Shimadzu, column: C18, 5 µm, 4.6mm diameter, 250 mm length, 30°C) with an electrochemical (EC) detector (L-ECD-6A, +0.6 V). The mobile phase [1] was pumped through the column at $1mLmin^{-1}$. A standard curve comprising of 10, 50, 100 and 500 nML^{-1} aliquots of adrenaline and noradrenaline was made to calculate exact sample concentrations. Standards were made from adrenaline hydrochloride (SIGMA) and L-noradrenaline bitartrate (Koch-Light Laboratories Ltd.) and were processed in the same manner as the samples.

Raw data was analysed using a one-way repeated measures analysis of variance (ANOVA) with Newman-Keuls post-test analysis. This allowed comparison of data points within a group and also comparison between groups. In all cases $P \leq 0.05$ was used to indicate a significant difference. Linear regression analysis was performed to investigate changes in dorsal aortic pressure relating to heart rate, haematocrit or catecholamine concentrations.

Series 2.2. Effects of deep anaesthesia and determination of plasma catecholamine half-life in Chinook salmon and Snapper.

[1] The mobile phase consisted of 7.941g tri-sodium citrate, 0.034g EDTA, 0.135g heptane sulphonic acid (detergent) dissolved in 900 mL of ddH_2O. Acetic acid was added until pH=5.00. Finally 100 mL methanol was added and the solution degassed.

Initially for experiments in this series using Chinook salmon, a 10 minute induction with 60ppm AQUI-S was chosen to induce deep anaesthesia. However, the large size of the fish (mean weight 2412.0 ± 105g, 1 SEM) made them difficult to handle and very active in the experimental boxes. This lead to the dorsal aortic cannula being pulled out by 3 fish during the 48-hour rest period before induction. To overcome this, the initial anaesthesia and surgery in which the fish were cannulated was designated as the induction event. Fish were anaesthetized in AQUI-S prior to surgery to maintain continuity with the series 2.1 inductions.

60ppm AQUI-S took over 25 minutes to produce a satisfactory surgical anaesthesia (cessation of gill movements). The fish also twitched during the first stages of surgery and began periodic weak ventilation. A major indicator of deep anaesthesia in fish is loss of skeletal muscle tone and therefore a higher concentration of 120 ppm AQUI-S was used to anaesthetize the fish. This achieved surgical anaesthesia within 10 minutes and prevented movement during surgery.

Chinook salmon (*Oncorhynchus tshawytscha)* were donated by Issac's Salmon Farm, and transferred to their on site hatchery in chilled, oxygenated water. They were stored in a large round indoor tank (1.5m diameter x 1.5m depth), with constantly flowing fresh water. Both the tank water and that used in the experiments came from the same source and had a constant temperature of 12°C.

Experiment 2.2.a Measurement of catecholamine release during deep anaesthesia and surgery and determination of catecholamine half-life in Chinook salmon blood.

When required, Chinook salmon (mean weight 2412 ± 105g, range 2097-2852g, n = 6) were removed from the holding tank with a net and placed in a bucket of 120ppm AQUI-S, until surgical anaesthesia was reached (gill ventilation had stopped). Fish were then placed ventral side up on an operating sling and a piece of cannula (1mm OD, 0.5mm ID, approximately 1 m long) was inserted into the dorsal aorta via the roof of the mouth using the same procedure as described in series 2.1. To secure the cannula further and prevent movement in the vessel, the cannula was secured via a single suture of surgical thread in the skin on the roof of the mouth. A single drop of 'superglue' (Sellys') was also placed over the opening to the nose cannula through which the dorsal aortic cannula had

been exteriorised. This prevented the fish from pulling out the cannula as described previously (see above).

Immediately after surgery, a 300µl blood sample was taken and replaced with an equal volume of freshwater teleost saline[2]. The fish was then placed in the same experimental containers as described in series 2.1 and covered with black polythene.

Dorsal aortic blood pressure (cm H_2O) and heart rate (beats per minute, bpm) were recorded at 10, 20 and 40 minutes post-recovery. Recovery was deemed to begin when the fish first resumed active (un-assisted) ventilation. Further blood pressure and heart rate recordings were taken over the next 4 hours post-recovery. Blood samples were also taken at these times to record haematocrit, mean cell haemoglobin concentration and plasma catecholamine levels. Blood samples were processed and analysed in the same manner as previously described in series 2.1. Raw data was analysed using one-way repeated measures ANOVA with Newman-Keuls post-hoc test. Linear regression analysis was performed to investigate changes in dorsal aortic pressure relating to heart rate, haematocrit or catecholamine concentrations. In all cases $P \leq 0.05$ was used to indicate a significant difference.

The effective half-life of plasma catecholamines present in salmon and snapper blood was calculated using a one-phase exponential decay model. The determination of half-life for both adrenaline and noradrenaline using this method was calculated in the commercial graphing and statistics package GraphPad Prism®, version 3 for windows (San Diego, California, USA).

Experiment 2.2.b Measurement of catecholamine release during deep anaesthesia and surgery and determination of catecholamine half-life in Snapper blood.

These experiments were carried out at Crop and Food Research Seafood division, facilities in Nelson, New Zealand. New Zealand snapper *(Pagrus auratus)* were obtained from and held at the Seafood Research unit in large ($6.1m^3$) darkened fibreglass tanks supplied with filtered seawater, maintained at a constant temperature of 11°C. Again,

[2] Masses for freshwater Teleost saline (gL^{-1}):

NaCl 7.305g
KCL 0.383g
$Na_2H_2PO_4.2H_2O$ 0.156g
$MgSO_4.7H_2O$ 0.232g
$NaHCO_3$ 2.520g
Glucose 1.000g
$CaCl_2.2H_2O$ 0.147g

120ppm AQUI-S was used to anaesthetise the fish. On average snapper took 20-25 minutes to reach surgical anaesthesia.

When required, New Zealand snapper (*Chrysophrys auratus*) (mean weight 861.3 ± 64.6g, (±1SEM) range 653.4-1056.8g, n=7) were removed from the holding tank with a net and placed in a bucket of 120 ppm AQUI-S until surgical anaesthesia was reached. Fish were then placed upright, angled on their right hand side and oxygenated 60ppm AQUI-S flushed over the gills continuously. The morphology of snapper made it impractical to cannulate the dorsal aorta. Therefore the afferent branchial artery was cannulated allowing measurement of ventral aortic blood pressure (VAP). The left operculum was pulled forward to expose the gill arches and the third gill arch isolated from the others. A small hole was made in the afferent branchial artery of the third gill arch with an 18-gauge needle. The hole was on the left hand side, immediately ventral to the gill lamellae and in the mid-section of the arch. The vessel was then occlusively cannulated with polyethylene cannula (0.86mmID, 1.27mmOD) and 1 ml of heparinized marine teleost saline[3] slowly injected into the fish. To further secure the cannula a ligature was tied around the cannula and the arch, taking care not to kink the cannula. A small blood sample (approx. 300µl) was taken at the time when the cannula was secured.

Fish recovered in darkened experimental boxes supplied with filtered seawater (flow rate approximately 1.5L per minute). Ventral aortic pressure (VAP) and HR were measured continuously throughout recovery.

Blood sampling regimes and processing were identical to that for Chinook salmon in experiment 2.2a., with the exception that plasma samples were frozen in liquid nitrogen for storage. Again, raw data was analysed using one-way repeated measures ANOVA with Newman-Keuls post-hoc test. Linear regression analysis was performed to investigate changes in ventral aortic pressure relating to heart rate, haematocrit or catecholamine concentrations. In all cases $P \leq 0.05$ was used to indicate a significant difference. The effective half-life of plasma catecholamines present in snapper blood was calculated using a one-phase exponential decay model. All statistical analysis were performed using the commercial graphing and statistics package GraphPad Prism®.

---

[3] Masses for Marine Teleost saline (gL^{-1}):

NaCl 10.00g
KCL 0.300g
$CaCl_2.2H_2O$ 0.225g
$MgSO_4.7H_2O$ 0.150g
Glucose 1.000g
HEPES 0.580g
NaHEPES 1.980g

Results

Series 2.1. Cardiovascular changes and haematology of Chinook salmon during and after anaesthesia.

Experiment 2.1. Measurement of cardiovascular and blood variables of Chinook salmon during recovery from anaesthesia.

Following anaesthesia with 60ppm AQUI-S, fish were observed to recover their righting reflex and response to external stimuli (i.e movement near the experimental boxes) within 5 minutes of restoration of the water flow. During the following 6 hours, some fish responded to the presence of people near the experimental box by increasing swimming movements and occasionally some fish would thrash for a few seconds. Mean data for dorsal aortic blood pressure (DAP) and heart rate (HR), are presented in Figure 2.2 a & b. Haematocrit (Hct) and mean cell haemoglobin concentration (MCHC) for all fish used in series 2.1 are shown in Figure 2.3 a & b. Table 2.1 gives numerical values for all variables, presented as means ± 1 SEM over a 5-minute period at every sample time. Mean plasma catecholamine concentrations before and after induction are shown in Figure 2.6. All values are given as means ± 1 SEM, with significance levels set at $P \leq 0.05$ unless otherwise stated.

Pre-induction DAP was 40.6 ± 3.3 cmH_2O. On induction there was a 16% decrease in dorsal aortic blood pressure (Figure 2.2a), which stabilised around 35cmH_2O in the last 90 seconds of anaesthesia. However, these changes were not significant throughout the course of the experimental period.

Prior to induction the mean pre-induction heart rate for all fish was 43.7 ± 3.3 beats per minute (Figure 2.2b). Throughout induction there was a steady increase in heart rate, which became significantly different from pre-induction values 3 minutes into anaesthesia.

After anaesthesia, there was an immediate rise in DAP with values in the first 5-minutes all significantly higher than during induction (Figure 2.2a). By 60-minutes

TIME (minutes)	DAP (cm H_2O)	HR (bpm)	Hct (%)	MCHC ($g.dL^{-1}$)	Adrenaline ($nmol.L^{-1}$)
-5 (pre-induction)	40.6 ± 3.4	43.7 ± 3.3	21.9 ± 2.63	30.31 ± 1.75	3.94 ± 0.49
0 (induction)	38.5 ± 2.8	54.6 ± 2.6			
5 (post induction)	49.8 ± 4.8*	49.4 ± 4.6	25.9 ± 2.44*	26.06 ± 1.49^	3.72 ± 0.34
60	47.8 ± 4.7	41.6 ± 4.4+	23.9 ± 2.03	24.84 ± 1.56	4.23 ± 0.53
180	40.8 ± 3.9	47.1 ± 3.6	21.8 ± 1.95	30.32 ± 2.25	2.58 ± 0.62
360	43.3 ± 3.1	50.1 ± 2.9	19.9 ± 1.95	30.90 ± 2.64	3.58 ± 0.45

Table 2.1. Mean values for dorsal aortic blood pressure (DAP), heart rate, haematocrit (Hct), and plasma adrenaline levels in Chinook salmon before and after 5-minute anaesthetic induction with 60ppm AQUI-S. Error values are ± 1 SEM. * indicates a significant difference ($P \leq 0.05$, n = 7) from pre-induction values. + represents a significant difference ($P \leq 0.05$, n = 7) from induction values. ^ represents a significant difference ($P \leq 0.05$, n=7) from values at 3 and 6 hours recovery. Noradrenaline values are not presented as they occurred at such low levels.

post-induction blood pressure had decreased to levels just above pre-induction values and stayed this way over the next 6 hours.

There was a gradual but steady drop in heart rate throughout the initial 5-minute recovery period, plateauing in the final 90 seconds to a mean value of 40.7bpm (Figure 2.2b). 60-minutes into recovery, heart rate was significantly lower than induction values, although over the next 6 hours values rose to just above pre-induction values.

Over most of the experiment there was no correlation between heart rate and DAP (Figure 2.4a). However, at 6 hours post induction there was a significant negative correlation between heart rate and DAP (Figure 2.4b, $r^2=0.63$, $P \leq 0.05$).

Haematocrit increased from a pre-induction value of 21.9 ± 2.7% (1 SEM, n = 7) to a post-induction value of 25.9 ± 2.4% (1 SEM, n = 7) (Figure 2.3a). Throughout recovery there was a correlation between dorsal aortic blood pressure and haematocrit ($r^2= 0.22$, $P \leq 0.01$, n=7, Figure 2.5) for all fish indicating the increased haematocrit was at least partly responsible for the rise in DAP seen after anaesthesia.

By the end of the recovery period, dorsal aortic pressure and haematocrit had fallen, returning to pre-treatment values (Figure 2.2a & 2.3a respectively).

During recovery, mean cell haemoglobin concentration (MCHC) decreased, although not significantly, from a pre-induction value of 30.31 ± 1.75 $g.dL^{-1}$ (1 SEM, n=7) to a post-induction value of 26.06 ± 1.49 $g.dL^{-1}$ (1 SEM n = 7) (Table 2.1 and Figure 2.3b).

Although there was some variability, plasma catecholamine levels did not rise significantly post-induction. Both adrenaline and noradrenaline levels remained below 5nmol.L^{-1} (Figure 2.6) and were not significantly different from pre-induction levels. Adrenaline concentrations exceeded those of noradrenaline in the plasma, which is a result typical of teleost fish (Randall and Perry, 1992).

Series 2.2. Effects of deep anaesthesia and determination of plasma catecholamine half-life in Chinook salmon and snapper.

Experiment 2.2.a Measurement of catecholamine release during deep anaesthesia and surgery and determination of catecholamine half-life in Chinook salmon blood.

Following anaesthesia with 120ppm AQUI-S all fish recovered their righting reflex and response to external stimuli within 5-8 minutes following restoration of water flow. However, several fish occasionally reacted to the presence of people near the experimental boxes by thrashing violently. Heart rate and DAP values following surgical anaesthesia are shown in Figure 2.7 a&b respectively. Haematocrit and MCHC data is shown in Figure 2.8 a&b.

DAP rose initially over the first 15 minutes of recovery from surgery before stabilizing at a mean of 65cm H_2O for the next 45 minutes (Figure 2.7b). By 120 minutes post-surgery, blood pressure was significantly lower ($P<0.01$) than during the first hour of recovery. DAP fell to 37cm H_2O for the final 2 hours of recovery.

Throughout the first hour of recovery from surgery, heart rate rose significantly from an initial mean value of 55bpm in the first 15 minutes to a maximum of 68bpm by 60minutes (Figure 2.7a). However, by 2 hours into recovery heart rate had returned to initial values, remaining at approximately 54bpm over the next 4 hours. For all fish at all

times throughout recovery there was no significant correlation between heart rate and DAP.

Plasma adrenaline and noradrenaline half-life data for Chinook salmon are presented in Figure 2.10 a&b. Representative graphs of catecholamine half-lives for an individual fish are presented in Figure 2.9. All fish showed an exponential decline in circulating plasma adrenaline and noradrenaline levels throughout recovery. The average concentration of adrenaline measured at surgery was 810.11 ± 172.46 nmol.L^{-1}, with maximum and minimum concentrations 1397.61nmol.L^{-1} and 332.78nmol.L^{-1} respectively. Despite the high plasma levels of adrenaline at surgery, the catecholamine was cleared rapidly, with the mean half-life for circulating plasma adrenaline in Chinook salmon blood being 9.27 ± 0.73 minutes (r^2=0.99, Figure 2.10a). The maximum calculated half-life was 11.21 minutes. There was no correlation between half-life and plasma adrenaline concentration at time of surgery, suggesting that the clearance mechanism was not saturated.

Plasma noradrenaline concentration followed the same pattern as adrenaline concentrations throughout recovery (Figure 2.10b). The main difference between the two catecholamines was the maximum concentration released. Noradrenaline was released at lower concentrations than adrenaline and had a similar plasma half-life. The mean half-life for noradrenaline in Chinook salmon blood was calculated at 11.33 ± 4.41 minutes (r^2=0.99, Figure 2.10b). The maximum plasma noradrenaline concentration measured at surgery was 300.36 nmol.L^{-1}, slightly less than the minimum concentration of adrenaline measured at surgery.

There was a strong positive correlation (r^2=0.67, P<0.0001, Figure 2.12) between plasma concentrations of adrenaline and noradrenaline released during surgery suggesting a concomitant release of the two catecholamines. Despite several fish struggling violently in the experimental boxes during the first hour of recovery, there was no evidence of further significant release of either adrenaline or noradrenaline.

There was no correlation between adrenaline or noradrenaline and DAP, heart rate or MCHC. There was however a weakly significant positive correlation between haematocrit and both adrenaline (r^2=0.24) and noradrenaline (r^2=0.21) (Figure 2.11a&b)

During the experiment, two fish pulled out their cannulae before the end of the 4-hour recovery period. Therefore statistics presented for haematocrit and MCHC after the

first hour of recovery represent a group of n=4. Results during the first hour represent a group of n=6.

Haematocrit dropped significantly over the first half-hour of recovery from a mean value of 40.7 ± 1.0% at surgery to 34.7 ± 1.0% by 30 minutes (Figure 2.8a). There was a slight increase in Hct at the end of the first hour, but by 120 minutes this had again dropped significantly below surgical values ($P<0.05$, n=4).

Mean cell haemoglobin concentration rose steadily throughout recovery from an initial value at surgery of 19.9 ± 1.2g.dL^{-1} through to a final value at 4 hours of 27.3 ± 0.79 g.dL^{-1} (Figure 2.8b). Haemoglobin concentrations during the first 40 minutes of recovery were significantly lower than final values at 4 hours recovery.

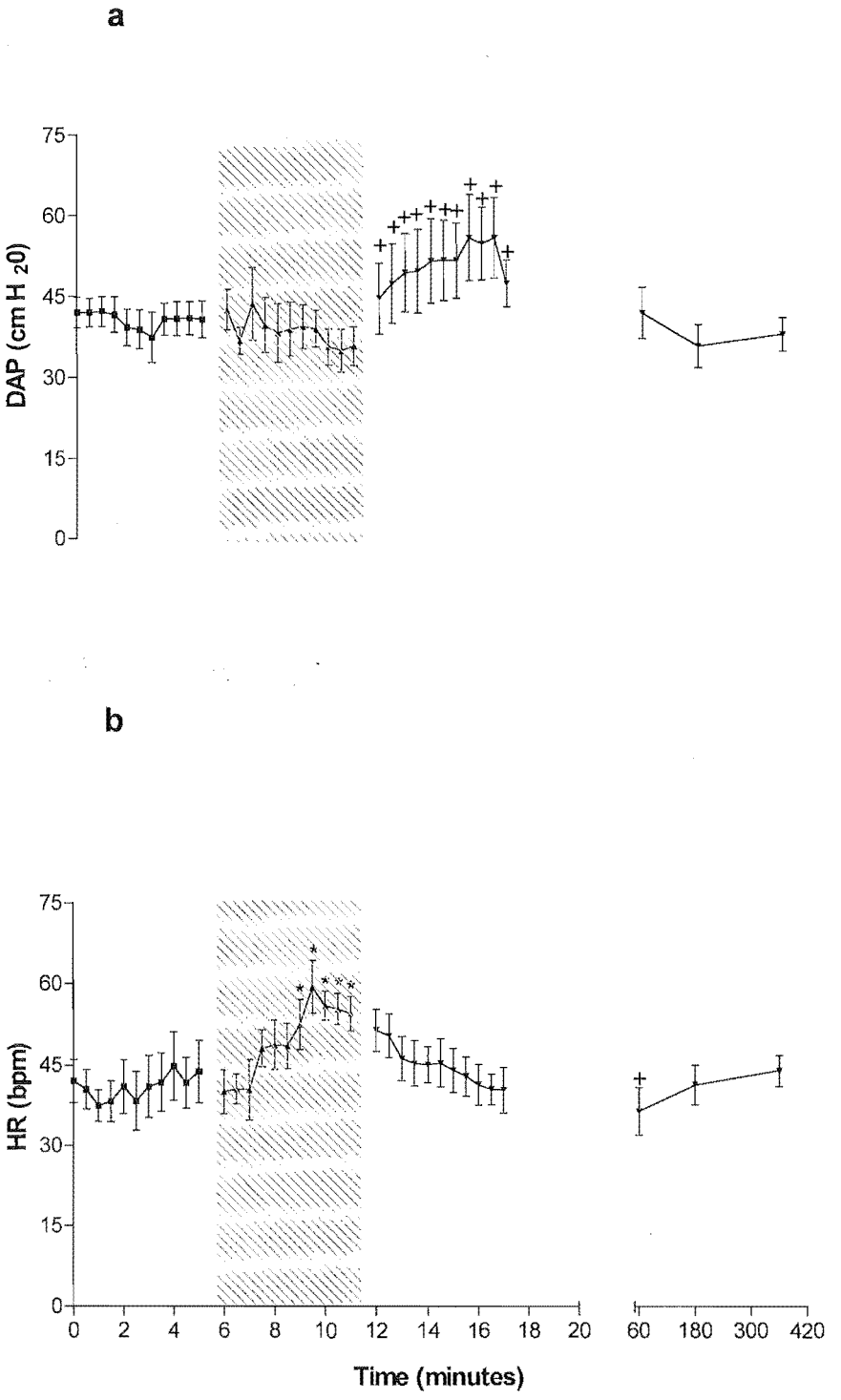


Figure 2.2 a&b. Graphs of dorsal aortic blood pressure (DAP,(a)) and heart rate (HR,(b)) of Chinook salmon before (■), during (▲) and after (▼) 5-minute induction with 60ppm AQUI-S. * Indicates a significant difference ($P \leq 0.05$, $n = 7$) from pre-induction values.+ Represents a significant difference ($P \leq 0.05$, $n = 7$) from induction values. Values are means ± 1 sem. Shaded area represents time of induction.

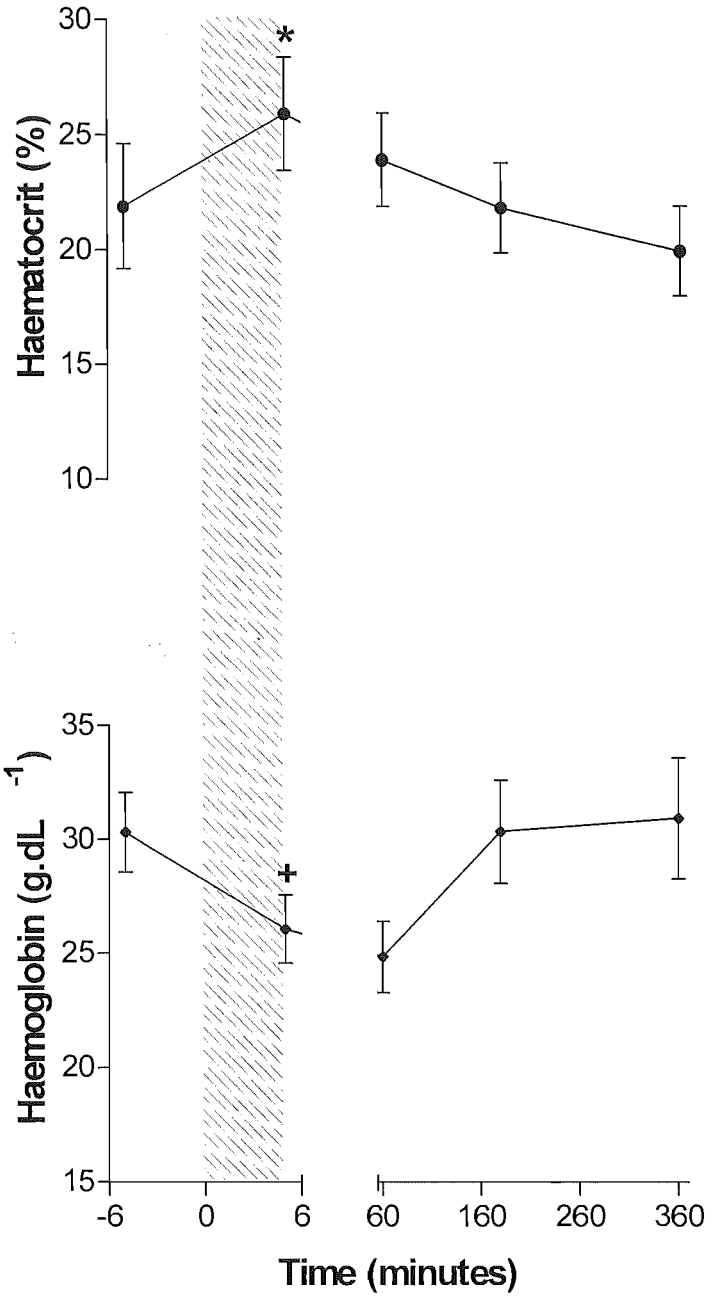


Figure 2.3 a&b. Haematocrit (Hct,%, (a)) and mean cell haemoglobin concentration (haemoglobin $g.dL^{-1}$, (b)) (means ± 1SEM) of Chinook salmon blood before and after 5 minute induction with 60ppm AQUI-S. * denotes a significant difference ($P \leq 0.005$, n=7) from pre-induction values. + denotes a significant difference from values at 3 and 6 hours recovery ($P \leq 0.005$, n=7).Shading indicates time of induction.

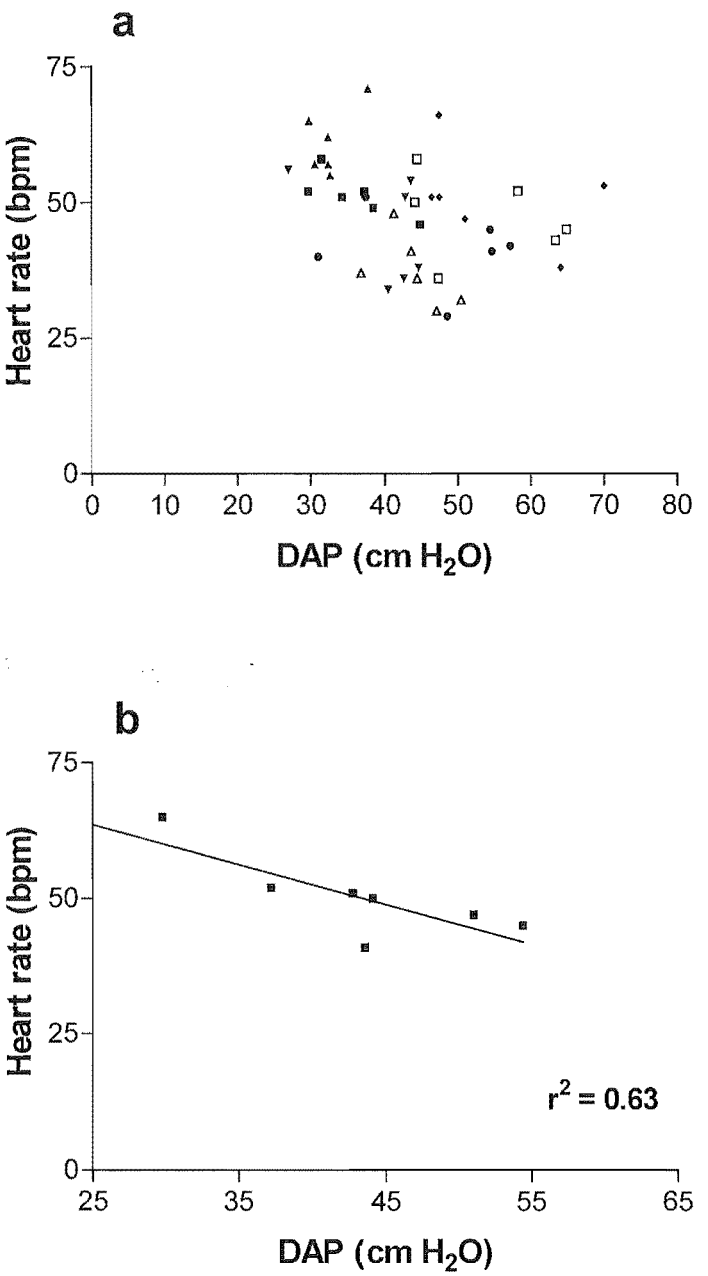


Figure 2.4 a & b. Graphs showing linear regression correlation between dorsal aortic blood pressure and heart rate in Chinook salmon during recovery from 5 minute induction with 60ppm AQUI-S. (a) If data for all fish (n = 7) at all times during recovery is considered, there is no correlation between DAP and heart rate. (b) At the 6 hour mark, there is a significant correlation ($r^2 = 0.63$, $P \leq 0.05$, n = 7) between DAP and heart rate indicating return of a functional baroreflex.

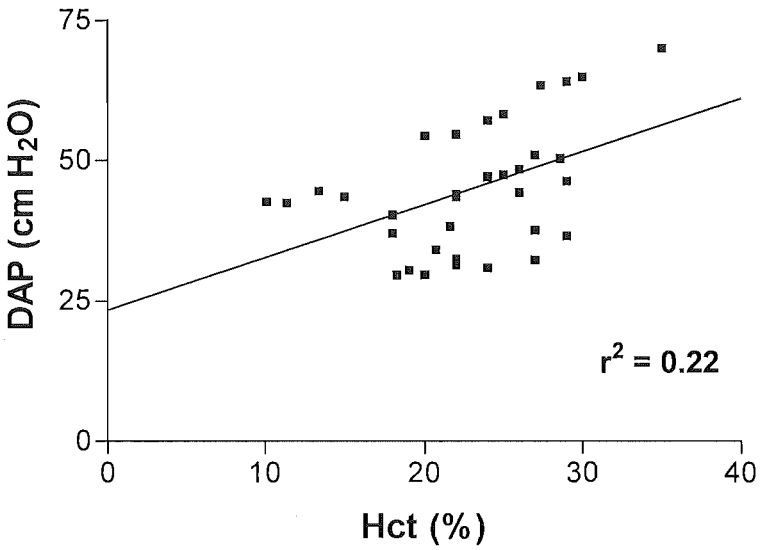


Figure 2.5. Regression correlation between dorsal aortic pressure (DAP) and haematocrit (Hct) ($r^2 = 0.22$, $P \leq 0.05$ n = 7) of Chinook salmon throughout recovery from 5-minute induction with AQUI-S.

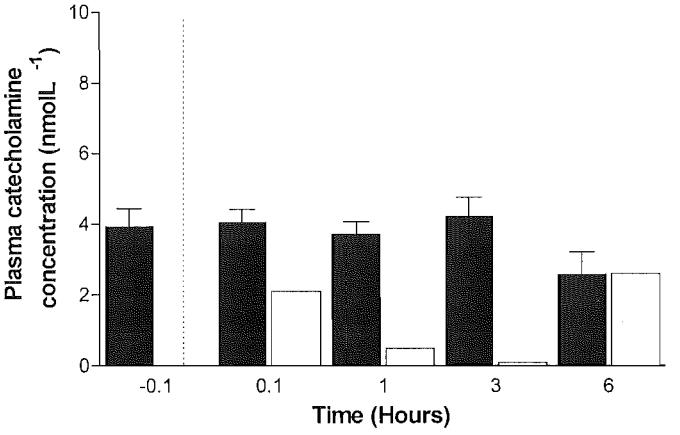


Figure 2.6. Graph of mean adrenaline (black columns, n = 7) and noradrenaline (clear columns, n = 7) plasma concentrations of Chinook salmon blood before and after 5-minute induction with 60 ppm AQUI-S. Error bars are ± 1 SEM. Dashed line (----) denotes time of induction. Note, noradrenaline values have no error bars as at any one time throughout induction and recovery, only one fish released noradrenaline at detectable levels. Therefore error calculation was impossible.

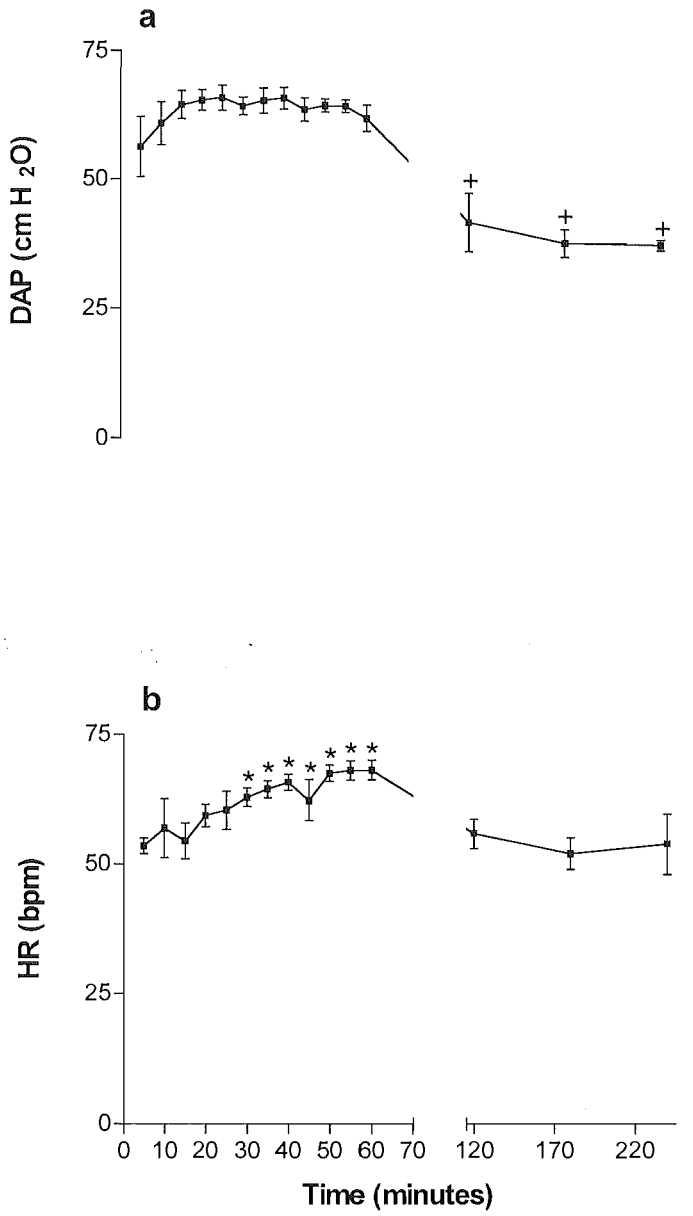


Figure 2.7 a&b. Graphs of dorsal aortic blood pressure (DAP) (a) and heart rate (HR) (b) of Chinook salmon following surgical anaesthesia with 120ppm AQUI-S. * Indicates a significant difference ($P < 0.001$, $n = 6$) from the first 15 minutes of recovery. + Represents a significant difference ($P < 0.01$, $n = 6$) from the first 60 minutes of recovery. Values are means ± 1 sem.

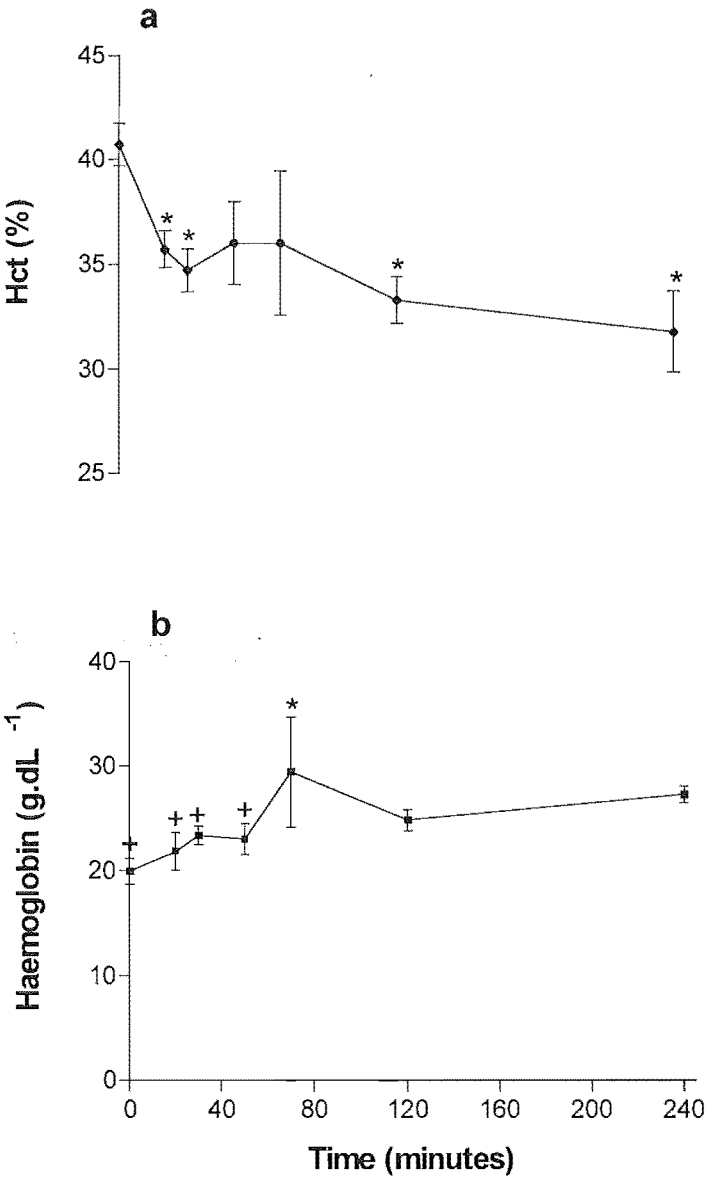


Figure 2.8 a&b. Haematocrit (Hct,%) (a) and mean cell haemoglobin concentration (b) of Chinook salmon blood following surgical anaesthesia with 120ppm AQUI-S. For both graphs, * denotes a significant difference ($P \leq 0.05$, n=7) from time zero (surgery) + denotes a significant difference ($P \leq 0.05$, n=7) from values at 240 minutes.

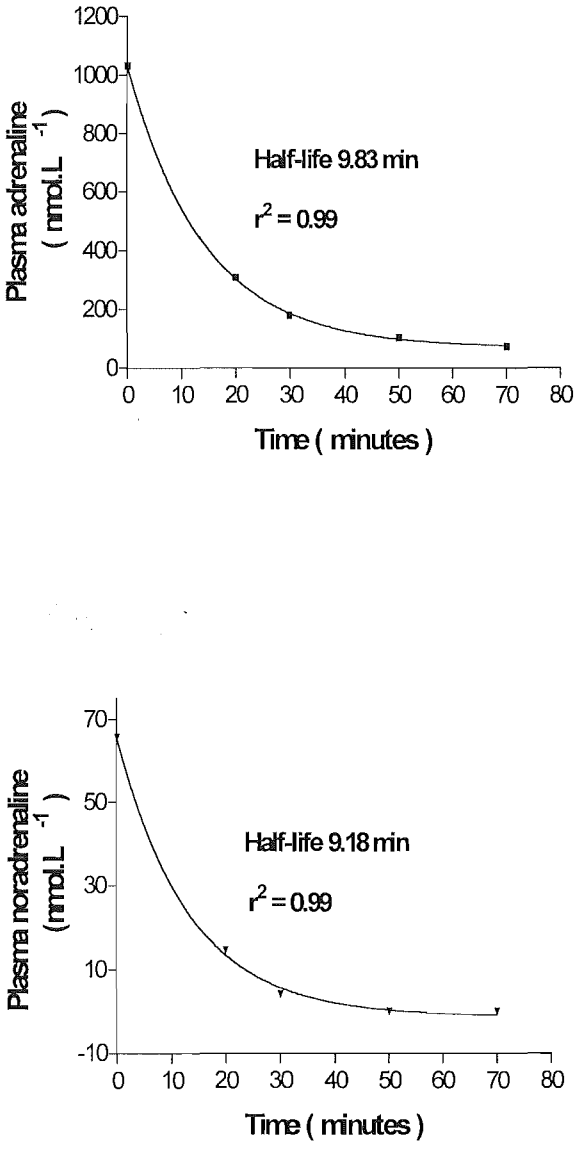


Figure 2.9 Representative graphs of adrenaline (■) and noradrenaline (▼) half-lives for a single chinook salmon used in series 2.2a. Half-life values are presented on each graph.

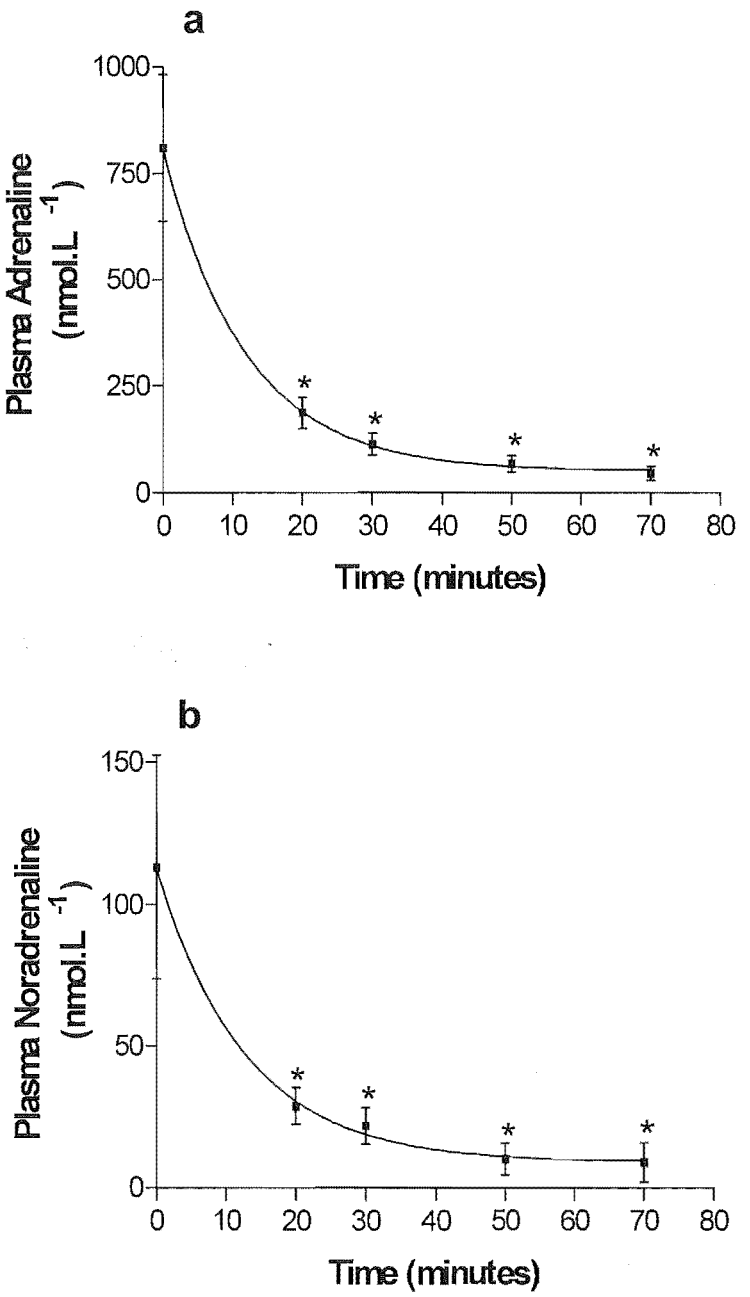


Figure 2.10 a&b. Graphs of mean plasma adrenaline (a) and noradrenaline (b) half-life in Chinook salmon blood following anaesthesia with 120ppm AQUI-S. Values are means ± 1 SEM. Mean half-life for plasma adrenaline over the first 70 minutes of recovery was calculated as 9.27 ± 0.73 minutes (n=7, r^2=0.99). Mean half-life for noradrenaline during the same period was calculated as 11.33 ± 4.41 minutes (n=7, r^2=0.99). * indicates a significant difference from values at time zero (surgery) for both catecholamines ($P < 0.001$).

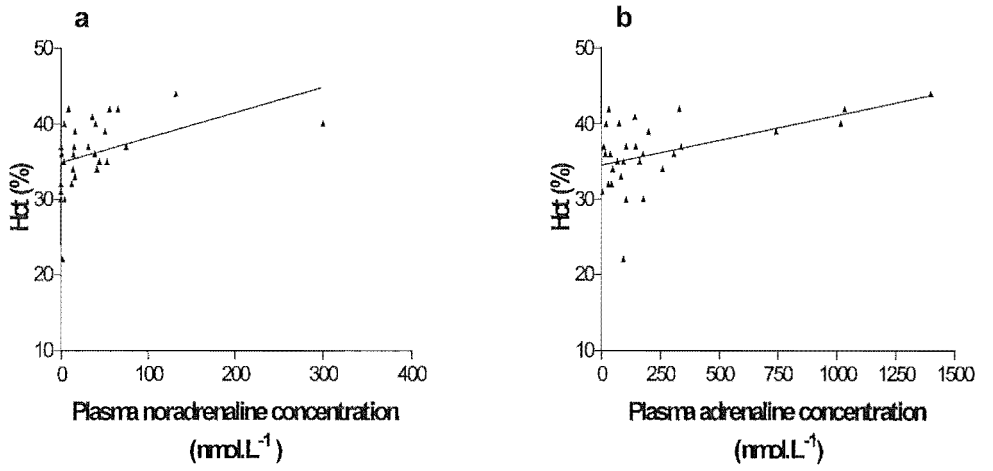


Figure 2.11. a&b. Regression correlation between (a) plasma noradrenaline and haematocrit (Hct) (r^2=0.21) and (b) plasma adrenaline and Hct (r^2=0.24) in Chinook salmon during the first 70 minutes of recovery from surgical anaesthesia with 120ppm AQUI-S.

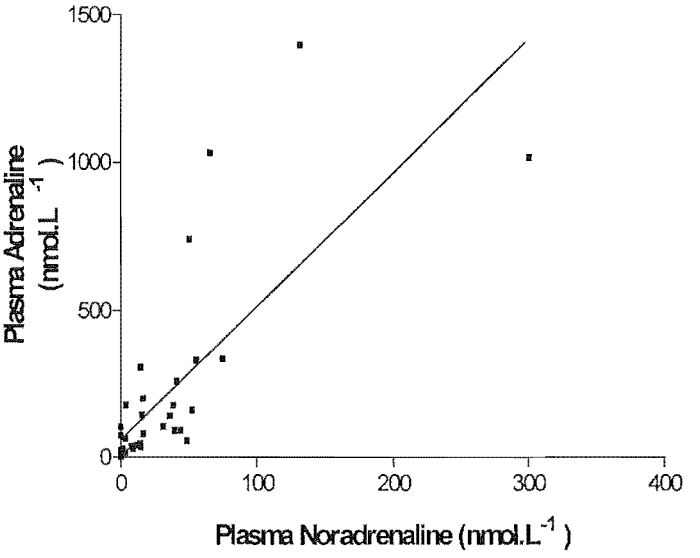


Figure 2.12 Regression correlation between circulating plasma adrenaline and noradrenaline concentration in Chinook salmon over the first hour following anaesthesia with 120ppm AQUI-S (r^2=0.67, P<0.001)

Experiment 2.2.b Measurement of catecholamine release during deep anaesthesia and surgery and determination of catecholamine half-life in Snapper blood.

Ventral aortic blood pressure and heart rate data for fish used in experiment 2.2b are presented in Figure 2.13a&b. Haematocrit (Hct) and mean cell haemoglobin concentration (MCHC) data can be seen in Figure 2.15a&b. All values calculated for the first 70 minutes of recovery are means ± 1 sem, n=7. In common with gill artery cannulation in some other species (e.g. *Salmo salar*, M.E. Forster, pers. communication) there was a rapid attenuation of the pulse pressure. This meant that reliable recordings of VA pressure in all 7 fish could only be made over the first two hours. However, blood samples were able to be collected via the cannula from 5 fish at later times, despite difficulties with pressure recordings. Therefore values for any given variable at 2 hours recovery represents n=5. VAP and heart rates are given only up to 2 hours recovery, whereas MCHC and Hct data is given up-to 4 hours recovery.

Following surgery, VAP rose steadily and significantly for the first 30 minutes of recovery from an initial mean value of 31.6 ± 4.9 cm H_2O to a maximum of 51.3 ± 4.9 cm H_2O by 28 minutes recovery (Figure 2.13a). Over the next 30 minutes VAP remained relatively stable, fluctuating around a mean value of 46.1 ± 1.2 cm H_2O. By 20 minutes into recovery, VAP was significantly higher than during the initial 15 minutes of recovery ($P < 0.001$, n=7) remaining this way for the next 2 hours.

Heart rate also rose steadily throughout the first half-hour of recovery to a maximum value of 49.6 ± 3.9 bpm by 28 minutes (Figure 2.13b) Indeed, there was a significant positive regression between VAP and heart rate throughout the first 70 minutes of recovery (r^2=0.29, P<0.001). At 70 minutes of recovery heart rate was 45.4 ± 4.2 bpm. This value was still higher than the initial heart rate immediately post-anaesthesia of 37.5 ± 4.4 bpm.

Haematocrit declined significantly throughout the first 70 minutes of recovery, from a surgery value of 31.0 ± 0.95% to 21.0 ± 0.81% by the end of the first hour. All values after 20 minutes were significantly lower than at the time of surgery (P<0.05, n=7, Figure 2.15a). By 2 hours into recovery, Hct had begun to recover rising to 23.0 ± 0.54% (n=5). The decline in Hct seen at 4 hours may be an artefact of one fish that whose Hct dropped by 5% from its value at 2 hours. If this fish is excluded from calculation of the

mean Hct value at 4-hours, the result is 23.2 ± 1.6% (n=4) which is almost identical to Hct at 2 hours recovery.

In contrast to the increase in Mean cell haemoglobin concentrations (MCHC) seen in Chinook salmon following surgery, snapper MCHC values were stable throughout the first 2 hours of recovery at about 21 $g.dL^{-1}$ (Figure 2.15b). The variation in MCHC seen at 30 minutes may be an artefact as one fish that at this time showed a drop in MCHC of 6$g.dL^{-1}$ compared to its previous value at 20 minutes. There was a rise in MCHC at 4 hours into recovery and although this represents only n=4 fish, all individuals showed an average increase in haemoglobin concentration of 3.04 ± 0.52 $g.dL^{-1}$ from values at 2 hours recovery.

Mean values for plasma adrenaline half-life in Snapper are shown in Figure 2.16a. Half-lives were calculated over the first 50 minutes of recovery from surgery. The mean half-life for circulating plasma adrenaline in snapper was calculated as 4.35 ± 3.26 minutes (n=7). As with Chinook salmon, there was an exponential decrease in plasma catecholamines throughout recovery, despite many fish struggling to escape confinement. By 20 minutes into recovery, plasma adrenaline levels were significantly lower than those measured at surgery ($P<0.05$, n=7). Plasma adrenaline values in snapper were considerably lower than those seen in Chinook salmon. The average plasma adrenaline concentration measured at surgery in snapper was 149.60 ± 61.85 $nmol.L^{-1}$, with maximum and minimum values 390.33 $nmol.L^{-1}$ and 37.02 $nmol.L^{-1}$ respectively.

Only one fish released detectable levels of noradrenaline throughout recovery.

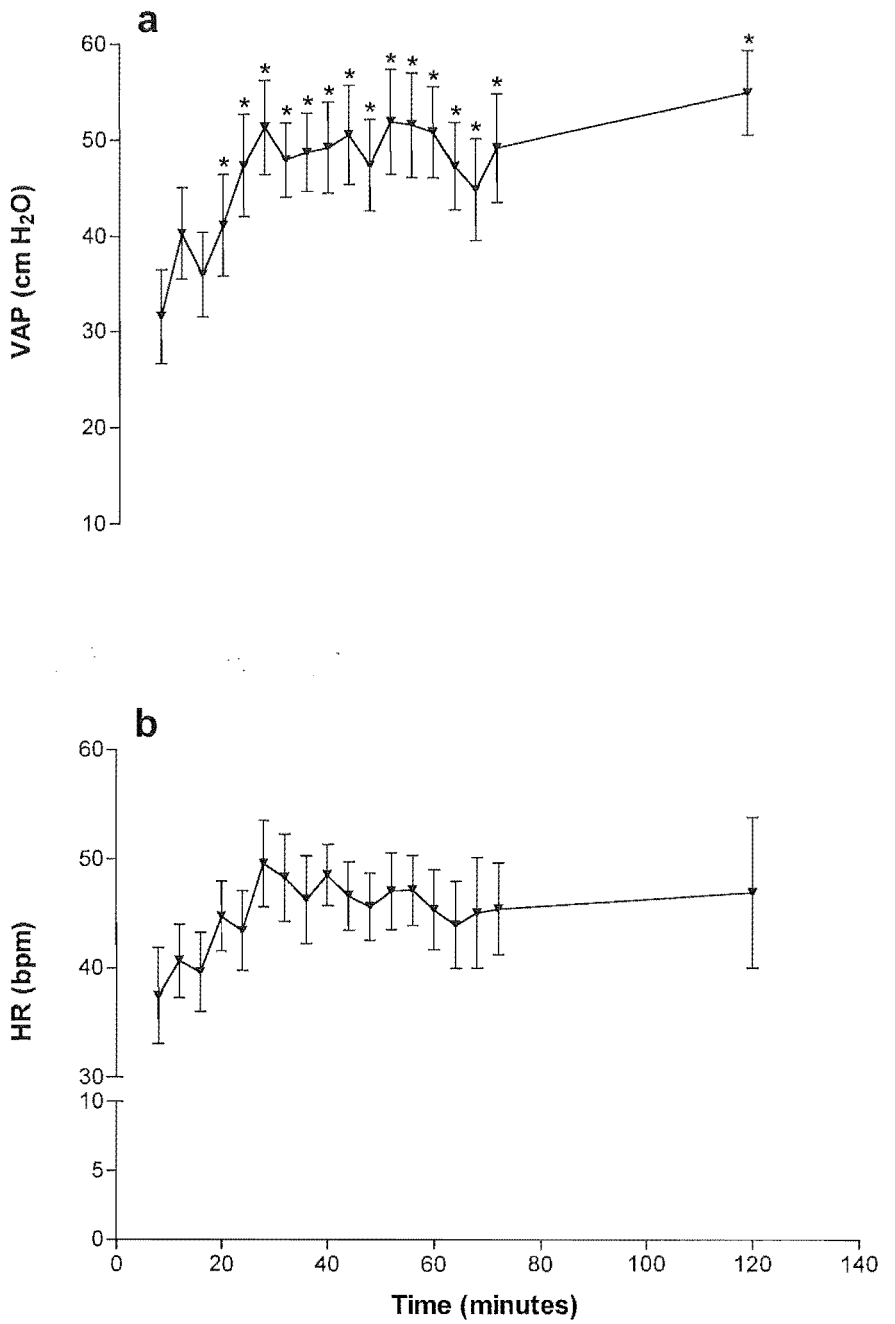


Figure 2.13.a&b Ventral aortic pressure (VAP) (a) and heart rate (b) following surgical anaesthesia with 120ppm AQUI-Sin the New Zealand Snapper *Pagrus auratus*. Due to difficulties with blood pressure and heart rate recordings, values are given only up-to 2 hours into recovery. Values are means ± 1 sem. * represents a significant difference ($P<0.001$, $n=7$) from values during the first 16 minutes of recovery. Values given at 2 hours recovery represent $n=5$

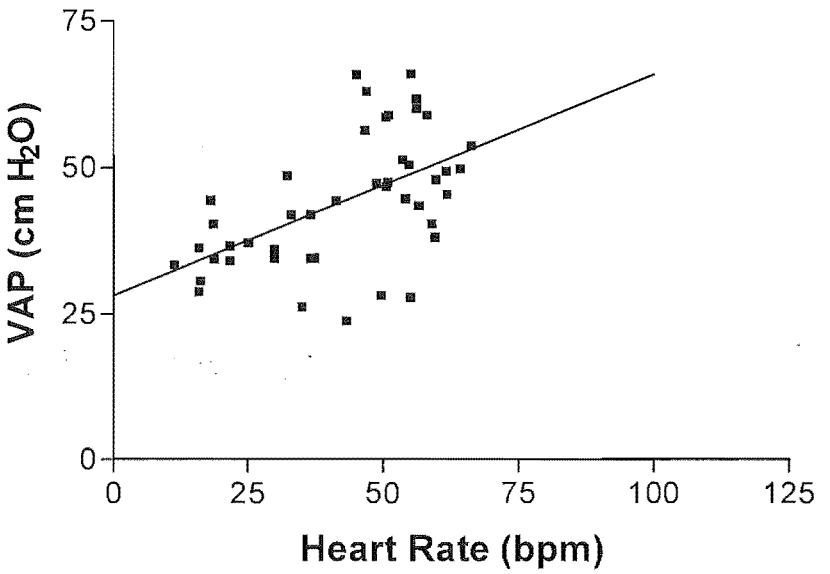


Figure 2.14 Regression correlation between ventral aortic blood pressure and heart rate during the first 70 minutes of recovery from anaesthesia with 120ppm AQUI-S in New Zealand Snapper (r^2=0.29, P<0.001, n=7)

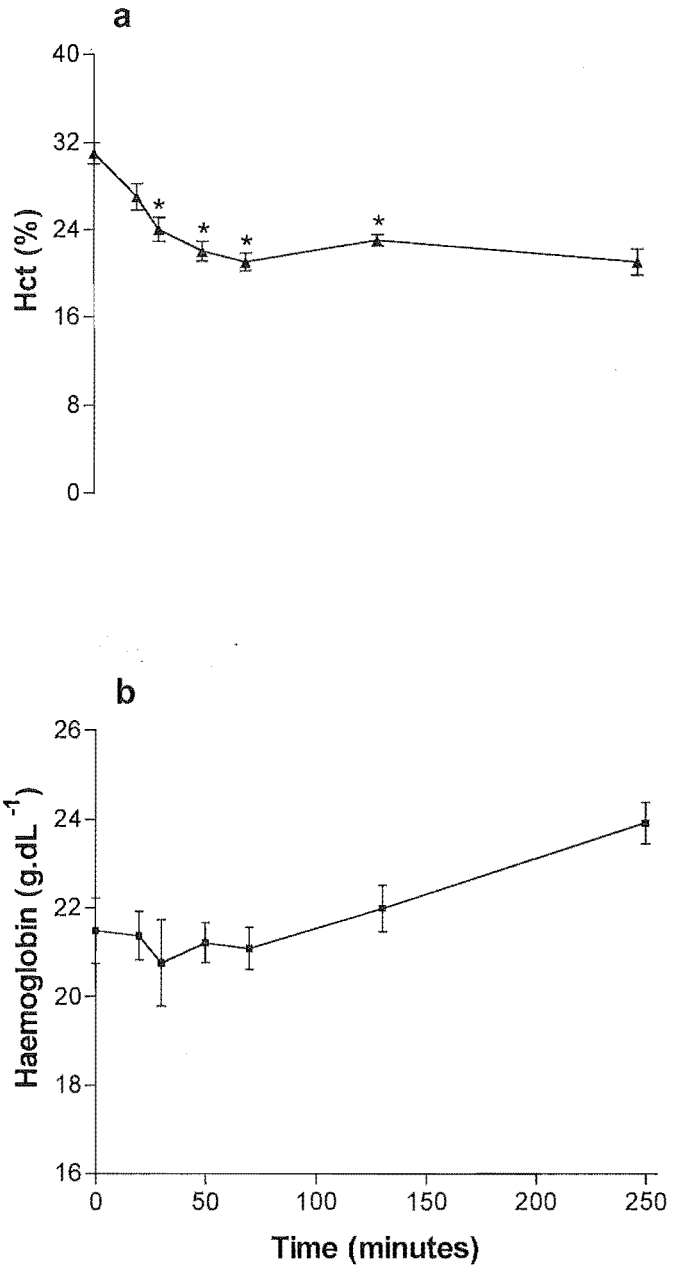
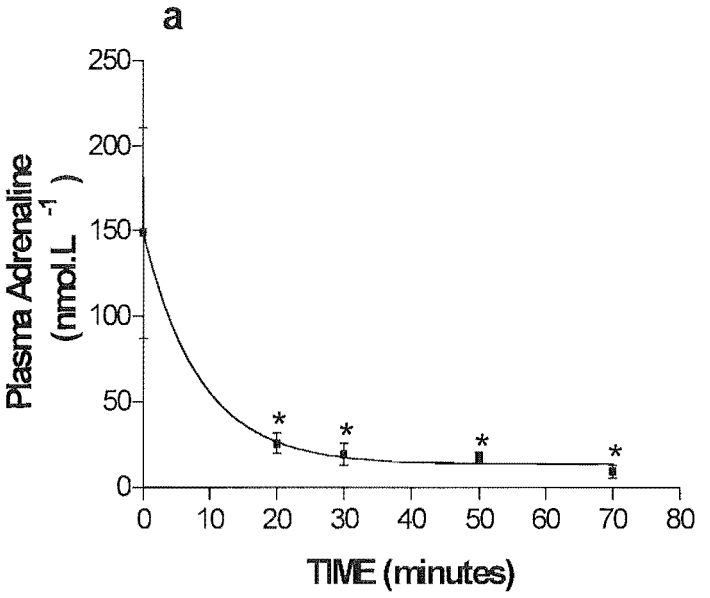


Figure 2.15 a&b. Haematocrit (Hct, a) and mean cell haemoglobin concentration (Haemoglobin, b) of snapper blood following surgical anaesthesia with 120ppm AQUI-S. *denotes a significant difference ($P \leq 0.001$, n=7) from time zero (surgery). Values for the first 70 minutes represent n=7. Values for 2 and 4-hours represent n=5.



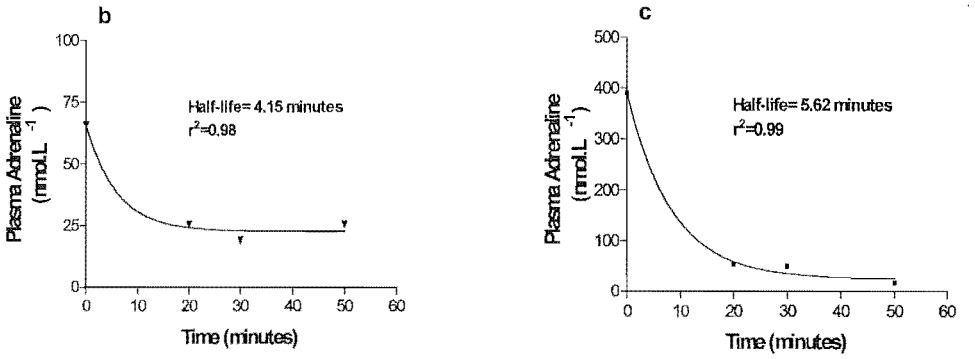


Figure 2.16 a-c. Mean half-life of plasma adrenaline in New Zealand snapper following surgical anaesthesia with 120ppm AQUI-S (a). Mean half-life over the first 50 minutes of recovery was calculated as 4.35 ± 3.26 minutes, n=7. Graphs (b) and (c) show plasma adrenaline half-lives for 2 individual fish used in series 2.2b., again over the first 50 minutes of recovery. Calculated half-lives and r^2 values appear on each graph.

Discussion

Changes in heart rate and blood pressure.

Response to light anaesthesia.

Published resting values for dorsal aortic pressure in the con-generic species *Oncorhynchus mykiss*, range from 41.9 cmH_20 (31.0mmHg) – 51.3cm H_20 (37.9mmHg) (Randall and Perry, 1992; Fredricks et al., 1993; Olson, 1998b), while resting heart rates range from 32bpm- 48bpm (Lochowitz et al., 1974; Olson, 1998b). Data for resting DAP and heart rate in Chinook salmon, gathered under almost identical conditions as those in the present study and in the same laboratory, give mean resting DAP and heart rate values as 46.0 cmH_20 (34.0mmHg) and 50.8 bpm respectively. Given that pre-induction values for those fish used in Experiment 2.1 fall at the lower end of published values and below those previously obtained in the same laboratory with the same species of fish, this would indicate that despite the invasive nature of the surgery and subsequent confinement in experimental boxes, the fish in Experiment 2.1 were relatively unstressed prior to induction. This is further supported by the low plasma catecholamine concentrations prior to induction.

Chinook salmon in the present study anaesthetised with 60ppm AQUI-S exhibited similar changes in heart rate and blood pressure to those seen in other species exposed to brief anaesthesia with MS222. In the rainbow trout, (Lochowitz et al., 1974; Soivio et al., 1977) tench, (Randall, 1962) several other teleost species (Healey, 1964; Randall and Smith, 1967; Houston et al., 1971a) and the spiny dogfish, (Peirce and Peirce, 1967), exposure to MS222 has been shown to elicit a transient increase in heart rate coupled with a fall in dorsal aortic blood pressure. Trout anaesthetised with either 40ppm or 100ppm MS222 exhibited a marked tachycardia above pre-induction values during the first 10 minutes of anaesthetic exposure (Houston et al., 1971a; Lochowitz et al., 1974). In the spiny dogfish, *Squalus acanthias,* MS222 has also been shown to decrease stroke volume and cardiac output (Peirce and Peirce, 1967). Prolonged exposure (>10 minutes) to MS222 in any of the above species resulted in an almost total collapse of cardiac function and the conclusion that MS222 in usual doses depresses cardiovascular function significantly. The authors also concluded the initial tachycardia seen is due to stress-induced catecholamine release, given that administration of beta-blockers such as DCI (dichloroisoprenaline) prior to anaesthesia abolishes the effect (Lochowitz et al., 1974).

Resting levels of adrenaline and noradrenaline have been measured in trout at between 1-5nmol.L^{-1}, with mean resting levels of salmon used in the present study measuring 3.94 ± 0.49 nmol.L^{-1} prior to induction (Table 2). In the present study, there was no significant increase in circulating catecholamines above 5nmol.L^{-1} during short-term exposure to AQUI-S anaesthesia. Therefore, it is unlikely that the increase in heart rate seen during induction in series 2.1 is due to humoral catecholamines exerting a direct excitory adrenergic stimulation on the salmon heart.

All vertebrate hearts, although myogenic, are under significant autonomic influence. The atria and junctional tissues are highly innervated receiving both parasympathetic (vagal cholinergic) and sympathetic (adrenergic) fibers (Baum, 1977). Although the distribution of adrenergic innervation may vary between species, in teleost fish the sinus venosus and atrium are typically well supplied with adrenergic nerves, particularly around the sino-atrial node, whereas parasympathetic fibers tend to predominate in the ventricle (Farrell and Jones, 1992). The target of these nerves are α and β adrenergic receptors expressed on the myocardial cells, although not necessarily all fish species express both receptors (Olson, 1998b). Whereas the β_2 receptors expressed in the skeletal muscle and vasculature are dilatory, the β_1 receptors of the heart are excitory and stimulation of adrenergic nerves leads to cardiac acceleration (Baum, 1977; Olson, 1998b). Sympatheic and vagal tone can however vary under certain circumstances. At rest vagal tone often pre-dominates, although under anaesthesia dogs have been shown to possess high sympathetic activity with relatively low vagal tone (Baum, 1977). An increase in the neural adrenergic tone on the heart may account for the increase in heart rate seen during anaesthesia in the present study. If this were the case, an increase in circulating plasma catecholamines would not be seen, as there would be no significant neural 'overflow' into the systemic circulation. The systemic vasculature also receives significant adrenergic innervation. In rainbow trout, resting systemic resistance is set by neurally mediated adrenergic tonus (Bushnell et al., 1992). Increased sympathetic activity would tend to act on α-adrenergic receptors increasing dorsal aortic blood pressure. This effect was not seen during induction with 60pm AQUI-S in the present study. Instead, there was a decrease in dorsal aortic pressure throughout induction with 60ppm AQUI-S. Low concentrations of AQUI-S have been shown to have a direct effect on both isolated blood vessels (Hill, 1999) and vascular beds causing a significant vasorelaxation that will be described in a future chapter. *In vivo,* direct anaesthetic effects on the vasculature may offset increased sympathetic activity to cause an overall reduction in blood pressure rather than an increase, which might otherwise be expected.

As well as the heart receiving adrenergic innervation, there is also a strong inhibitory cholinergic influence exerted by the parasympathetic fibres of the vagus nerve. This inhibitory branch of the vagus carries small myelinated cholinergic efferent fibers, which stimulate muscarinic cholinoceptors. The vagus also carries large myelinated fibers, thought to be sensory cholinergic afferent fibers (Farrell and Jones, 1992). *In vivo* the intrinsic pacemaker rate is generally much higher than the so-called 'resting rate' and is modified by autonomic activity. The resting rate therefore is a compromise between adrenergic stimulation and cholinergic inhibition (Farrell, 1981; Farrell and Graham, 1986; Randall and Perry, 1992; Olson, 1998b). In the cod, *Gadus morhua* the cholinergic and adrenergic tonus on the heart at rest are approximately 38% and 21% respectively. However, following exercise, cholinergic influence falls to 15% whereas adrenergic tonus increases to 28% (Axelsson, 1988). This response to exercise is seen in most teleost fish and it is likely that in many species studied, sympathetic excitation combines with a ' vagal release' to speed the heart (Satchell, 1991). However, between species there is variation in cholinergic tonus, e.g trout appear to be under a lesser extent of vagal inhibition than the cod (Satchell 1991). Even so, it is reasonable to assume that there is some degree of cholinergic influence on the intrinsic rate in both trout and the con-generic salmon species.

Anaesthetics act by widespread depression of the central nervous system and in mammals have been shown to have direct effects on a variety of ion channels including potassium and calcium channels (both voltage and ligand sensitive) glutamate receptors, 5HT receptors and $GABA_A$ channels (Trudell and Bertaccini, 2002). In all species so far studied, non-immobilizing anaesthetics (i.e local anaesthetic agents) primarily target the transmembrane protein regions of sodium ion channels and the nicotinic acetylcholine receptor family (Ragsdale et al., 1994; Trudell and Bertaccini, 2002). Anaesthetic binding blocks these channels preventing initial rapid depolarisation of nerve and muscle cells. Indeed, in mammalian studies, all classical volatile anaesthetics have been shown to reduce acetylcholine release, supporting the idea that cholinergic cell suppression is at least one part of anaesthetic action (Shipton, 1999). This action would inhibit the cholinergic branch of the vagus nerve innervating the fish heart, initiating a 'vagal release' during anaesthesia. This effect could explain the tachycardia seen during the initial stages of anaesthesia during induction with 60ppm AQUI-S in the present study. However, Hill (1999) found that AQUI-S produced significant effects on vagal nerve conduction in Chinook salmon only when very high anaesthetic concentrations were used. No significant chronotropic or inotropic effects were seen using 60ppm AQUI-S in isolated nerve preparations.

During the last 90 seconds of anaesthesia with 60ppm AQUI-S, salmon heart rate began to decline. Studies with MS222 on ventricle strips of rainbow trout (*Oncorhynchus mykiss*) have shown a direct effect of anaesthetic on the force of contraction (Randall, 1962; Peirce and Peirce, 1967; Ryan et al., 1993). However, given the relatively low concentration of anaesthetic used and the short period of anaesthesia in Series 2.1 it is unlikely blood concentrations of the anaesthetic would have reached a level great enough to have a direct effect on the heart muscle. In the Chinook salmon, AQUI-S has no significant chronotropic or inotropic effects on the heart except at concentrations 100 times higher than that required to produce anaesthesia in the whole animal (Hill, 1999). It is possible that the decrease in heart rate is due to a developing hypoxemia in the animal. Hill (1999) found that some AQUI-S anaesthetised Chinook salmon showed a significant decrease in blood PO_2 after a 5-minute induction. This was due mainly to a decrease in the rate and amplitude of ventilation, leading to a state of hypoxemia and initiating a hypoxic bradycardia. It is possible that this is the case in the present study.

As recovery begins, any effect of hypoxia on the heart rate would be short-lived as blood PO_2 increases. The continuing decrease in heart rate throughout recovery probably reflects the return of normal vagal cholinergic tonus on the heart coupled with a reduction in sympathetic tone. This is supported by the fact that heart rate does not fall below resting values during recovery which might be expected if the fall was due to a hypoxic bradycardia.

The drop in heart rate may also be an attempt to offset the significant increase in dorsal aortic blood pressure (DAP) seen at the start of recovery. DAP may rise for several reasons. If there was a direct vasodilatory effect of the anaesthetic on the vascular smooth muscle of the gill, then DAP might rise as branchial vascular resistance fell. This effect would be reduced as the anaesthetic is removed from the circulation. An increase in neural adrenergic tone (see above) could also increase blood pressure. The primary site of anaesthetic removal from the circulation is likely to be the gill. Blood flow through the gill is dependent on a number of factors and can be modified both humorally and neurally. In fish, the gills receive the entire cardiac output and flow is dependent on intrinsic factors such as ventral aortic pressure and dorsal aortic pressure. Ventral aortic pressure in turn is determined by cardiac output, which is the product of heart rate and stroke volume (Satchell, 1971; Soivio and Hughes, 1978; Olson, 1991; Satchell, 1991; Olson, 1998b). As heart rate drops, filling time increases and a rise in end diastolic volume will increase stroke volume (as per the Frank-Starling "Law of the Heart") (Satchell, 1971; Satchell, 1991; Olson, 1998b). This will raise systolic pressure in the ventral aorta and the

recruitment of the gill lamellae would be beneficial to the recovering animal in two ways; it would increase the gill surface area for optimal oxygen uptake following hypoxia and allow for a more rapid removal of the anaesthetic from the blood stream. Given that cardiac output and gill resistance were not recorded in this study, the above cardiovascular changes are inferred from gathered data rather than being quantified. However, Hill (1999) found that under almost identical experimental conditions and protocols, there was a significant increase in both cardiac output and stroke volume in the Chinook salmon following light anaesthesia with 60ppm AQUI-S. Studies examining changes in gill circulation during MS222 anaesthesia in the rainbow trout have shown that vasodilation of the secondary lamellae occurs during induction and continues even through the recovery stage (Soivio and Hughes, 1978). Normally, branchial vascular resistance increases during acute hypoxic exposure, which should act to minimise the effects of ion and water loss across the gill during a stressed state (Soivio and Hughes, 1978; Satchell, 1991; Randall and Perry, 1992; Sundin and Nilsson, 1997). It has been suggested that this vaso-constriction is mediated in part by acetylcholine and in part by a non-adrenergic, non-cholinergic (NANC) substance such as serotonin, acting on 5HT receptors. As previously stated, local anaesthetics tend to target these types of receptors causing an effective blockade (Ragsdale et al., 1994; Shipton, 1999; Trudell and Bertaccini, 2002). Therefore this might explain a continuing relaxation of the gill vasculature through at least the initial stages of recovery, particularly if the gill is exposed to large quantities of anaesthetic loaded blood from the tissues.

Recovery from Surgical anaesthesia and cannulation.

The response to hypoxia varies between fish species. In teleost fish, hypoxia elicits a significant increase in both ventral aortic pressure (P_{VA}) and dorsal aortic pressure (P_{DA}) in most fish species studied (Holeton and Randall, 1967; Fritsche, 1990; Fritsche and Nilsson, 1990; Satchell, 1991). This increase in blood pressure continues during recovery and is often coupled with a significant cardio-acceleration following the hypoxic period. Holeton and Randall (1967) found that in the rainbow trout, changes in P_{VA} were more marked than P_{DA} post-hypoxia and there was a marked reduction in pulse pressure upon return to normoxic water in both vessels. Both Chinook salmon and snapper exhibited elevated heart rate and blood pressure upon recovery from prolonged anaesthesia and surgery (P_{DA} in salmon, P_{VA} in snapper). Measurement of these variables prior to anaesthesia was not possible as cannulation surgery was included as part of the experimental protocol. However, it is possible to attempt to extrapolate the changes in

cardiovascular variables during anaesthesia by looking at the data gathered during recovery.

Blood pressure in salmon was high at the start of recovery and remained elevated throughout the first hour of recovery. This suggests the maintenance of hypoxia-induced hypertension as seen in other species. Heart rate was also high at the start of recovery and continued to rise over the next hour. This initially high value may be an artefact of sampling time as recovery was deemed to begin when unassisted ventilation began. It is possible that had sampling time been earlier, then the final stages of hypoxic bradycardia may have been observed more clearly than present starting values indicate. However, in the trout the introduction of fresh oxygenated water after a hypoxic exposure tripled the observed heart beat within 2 or 3 beats (Holeton and Randall, 1967).

In snapper, P_{VA} rose significantly throughout recovery after initially being low. Although there was a trend towards an increase in heart rate during recovery, this was not statistically significant due to large individual variability. In the Atlantic cod *Gadus morhua*, a significant increase in both dorsal and ventral aortic pressure in response to hypoxia is due to increased adrenergic nervous activity, with circulating plasma catecholamines augmenting the adrenergic tonus of the systemic vasculature (Fritsche and Nilsson, 1989). The high concentrations of circulating catecholamines measured in both salmon and snapper in Series 2.2 are most likely responsible for the elevation of blood pressure observed in both species. Even though the half-life for circulating plasma catecholamines in both species was determined to be less than 10-minutes, adrenergic tonus on the heart and vasculature may endure for some time post-hypoxia. Also, the effects of high concentrations of plasma catecholamines may be prolonged relative to their disappearance from the circulation (S.Egginton, pers.comm.). Circulating catecholamines may affect sympathetic neuronal activity in two ways; by activating pre-synaptic β-adrenoreceptors, in turn stimulating neural catecholamine release or by increasing the pool of catecholamines available for neuronal uptake and release (Xu and Olson, 1993a). Concentrations of adrenaline as low as $1x10^{-9}$M have been shown to prevent fatigue of the splanchnic nerve in perfused preparations (Xu and Olson, 1993a). The teleost vasculature is well innervated by adrenergic neurons and expresses a vast pool of both α and β adrenoreceptors (Randall and Perry, 1992). Blood flow through peripheral vessels is primarily regulated by vasomotor nerves to the trunk vessels. α-receptors predominate in the trunk vasculature and when stimulated by elevated plasma catecholamine concentrations, exert a vasoconstriction on the vessels, causing an increase in central blood pressure (Davie, 1981). As previously suggested, a direct vaso-relaxant effect of the

anaesthetic may combine with a depression of the CNS to cause a fall in aortic pressure during anaesthesia. As the anaesthetic is removed from neuro-muscular binding sites during recovery, the combined effects of increased neural and humoral catecholamine concentrations may be responsible for a rise in blood pressure. Both species of fish (salmon and snapper) would also be paying a significant oxygen debt due to the cessation of ventilation during anaesthesia (Hill, 1999). This debt would be greater in the snapper given that surgery took sometimes up to three times longer to complete than in the salmon experiments. This was due to the difficulty of the surgery and problems with blood clotting even when heparin was used. A higher ventral aortic blood pressure during recovery should move more blood into the gill lamellae for re-oxygenation and possibly reduce recovery time.

The low P_{VA} seen in snapper at the beginning of recovery is suggestive of a fall in ventral aortic pressure during anaesthesia. The eel-pout (*Zoarces viviparous*) exhibited a decrease in P_{VA} during a 5-minute hypoxic exposure, coupled with a typical hypoxic bradycardia (Fritsche, 1990). However, in contrast to other species there was no significant increase in blood pressure on return to normoxia, although there was a rise in heart rate. Treatment with atropine (a muscarinic cholinoceptor agonist) prevented hypoxic hypotension in *Z.viviparous* and at the same time provoked a positive chronotropic response on the heart (Fritsche 1990). Similar results have been reported for elasmobranchs. Hypoxic hypotension in the dogfish *Scyliorhinus canicula* is likely due to a reduction in cardiac output, since atropine treatment abolishes the response (Butler and Taylor 1971, cited in Fritsche 1990). Given the significant positive correlation between heart rate and P_{VA} in snapper throughout recovery, it is possible that in this species also, a drop in cardiac output due to a reduced heart rate is responsible for a hypotension during anaesthesia. As noted previously P_{VA} is dependent on heart rate. Negative chronotropic (and possibly inotropic) effects of the anaesthetic and subsequent hypoxia on the snapper heart would reduce cardiac output and P_{VA}. This relationship between P_{VA} and heart rate also explains a component of the rise in P_{VA} seen during recovery in snapper.

In Chinook salmon there was no correlation between heart rate and P_{DA} during recovery from deep anaesthesia, although both tended to rise. The significant rise in heart rate following anaesthesia and surgery is most likely due to the effect of high plasma catecholamine concentrations coupled with increased neural adrenergic tone. In the present study, salmon exhibited a significant negative correlation between heart rate and P_{DA} six hours following 5-minute induction with AQUI-S. This might indicate the return of a functional baroreflex following anaesthesia. This result was not seen in fish that had

undergone deep anaesthesia with 120ppm AQUI-S. Heart rate remained relatively high (when compared to similar sized fish used in previous experiments) in these fish for at least 4 hours following induction. This suggests a long-term effect of high catecholamine concentrations on the heart and may be related to catecholamine accumulation and degradation (see following discussion).

The different relationships between heart rate and blood pressure in salmon and snapper may reflect differences in the way the two species regulate blood pressure in the face of environmental hypoxia. Fresh water adapted chinook salmon might be expected to encounter hypoxic conditions at some stage during their life-cycle whether due to fluctuations in water temperature, exposure to environmental toxins or living in land locked habitats. Snapper however, are a marine teleost and as such are far less likely to encounter periods of even moderate hypoxia. Therefore differences between the two species with regards mechanisms to control blood pressure during hypoxia might not only be expected, but also a reflection of their natural environments.

Properties of the blood during anaesthesia.

In all experiments there was a rise in haematocrit (Hct) whether compared to pre-anaesthesia values (where measured) or published normal values for either salmon or snapper. Hct in Chinook salmon has been previously measured at 25-30% (Hill, 1999) while values for snapper have been published to range anywhere from 26-54% (Canfield et al., 1994). In salmon, the decrease in Hct during recovery was coupled with an increase in mean cell haemoglobin concentration (MCHC) in both Series 2.1 and Series 2.2. Although snapper showed an elevated Hct at the start of recovery, MCHC remained relatively stable throughout the first 50 minutes of recovery, before rising gradually over the next 4 hours. Haematocrit is defined as the proportion of red blood cells expressed as a percentage of a centrifuged sample of blood (Satchell, 1991). Blood viscosity is related to Hct and therefore so is vascular resistance. Because the heart must generate blood pressure to overcome vascular resistance, the blood pressure is also in turn related to Hct. This is illustrated in Figure 2.5. which shows a significant positive correlation between P_{DA} and Hct in salmon anaesthetised in 60ppm AQUI-S.

Hct and haemoglobin (Hb) content of the blood (of which MCHC is an effective measurement) tend to be correlated:- few red cells means there is less haemoglobin in the blood. During hypoxia an accumulation of CO_2 in the blood gradually decreases blood pH due to the hydration of CO_2 to bicarbonate and protons. This decrease in blood pH the

affinity of Hb for oxygen. This is known as the Bohr effect. Fish blood also exhibits a marked Root effect whereby the ability of the blood to become fully saturated, regardless of blood oxygen content, is significantly impaired in the face of low pH. Some fish have been shown to have such a significant Root effect blood that their blood cannot become fully saturated even at pressures as high as 100atm (Satchell, 1991). Trout and salmon blood are well known to exhibit a Root effect but the ability of salmonid erythrocytes to swell acts to offset the effects of pH on Hb-O_2 binding (Nikinmaa, 1990). The exact mechanisms of erythrocyte swelling in fish will be discussed in detail in the following chapter. However, a brief outline is offered below. When ventilation is depressed, e.g during anaesthesia, CO_2 accumulates in the blood, lowering intracellular pH. Under normal physiological conditions, the red blood cell (rbc) is more acidic and has a lower Na^+ concentration than the plasma (Fievet and Motais, 1991). However, a net increase in proton concentration in the rbc stimulates the activation of a Na^+/H^+ exchanger expressed on the cell membrane. When the exchanger is activated, Na^+ enters the cell down its electrochemical gradient and is exchanged in equal numbers with protons. To allow the cell to remain electrochemically neutral, chloride ions enter the erythrocyte *via* the HCO_3^- / Cl^- anionic exchanger. The net result is the transport of NaCl into the rbc coupled with osmotic water entry. Hence, the cell swells (Fievet and Motais, 1991; Nikinmaa and Salama, 1998). The advantage of swelling is that it effectively dilutes organic phosphates (in fish, primarily GTP) that competitively and strongly bind to Hb (Nikinmaa, 1990). This is reflected by a reduction in MCHC as the Hb has effectively been diluted as the cell swells. This form of erythrocyte swelling is non-adrenergically mediated.

In teleost fish, the liberation of catecholamines into the circulation can also initiate erythrocyte swelling *via* β-adrenergic stimulation of the Na^+/H^+ antiporter. Hydrogen ions are pumped out of the cell and the plasma becomes acidified, while the interior of the red blood cell becomes alkaline. Again water follows by osmosis and the cell swells (Satchell 1991; Gilmour, 1998). Both these mechanisms maintain oxygen binding and offset the Root effect of teleost blood. Erythrocyte swelling effectively increases the Hct due to the increased volume of the red blood cells relative to plasma. Hct can also be elevated by directly increasing the number of red blood cells entering the circulation. This is mediated via α-adrenergic contraction of the capsule of the spleen, which contains a rich store of red blood cells (Nilsson et al., 1976). In hypercapnic trout, this contraction is dose dependent in relation to circulating plasma catecholamines (Perry and Kinkead, 1989). In teleost fish, splenic contraction is the primary cause of arterial blood oxygen increase during hypoxemic induced elevation of circulating catecholamines (Randall and Perry, 1992).

Given the low concentrations of plasma catecholamines measured in Series 2.1 it is likely that erythrocyte swelling was due to respiratory hypoxia and CO_2 accumulation in the blood. It is not attributable to direct adrenergic stimulation of the Na^+/H^+ antiporter on the red blood cell. However, in series 2.2, red blood cell swelling in salmon blood occurs in the presence of high concentrations of both adrenaline and noradrenaline. In this case, it is probable that blood cells were sequestered from the spleen and then underwent adrenergically mediated cell swelling. In salmon, hct at the start of recovery from surgical anaesthesia was 42%, whereas it peaked at only 26% following light anaesthesia.

In snapper however, the high Hct seen post-anaesthesia was not coupled with a proportionally low MCHC. In fact, MCHC remained relatively stable over the initial hour of recovery before gradually increasing as Hct decreases. Again this is indicative of splenic contraction during induction and release of red blood cells into the circulation, rather than a significant red blood cell swelling. In a number of teleost species, including tench and carp, the Na^+/H^+ antiporter is not influenced by catecholamines (Koldkjær Knudsen and Jensen, 1998) and snapper may be included in this group.

Clearance of circulating plasma catecholamines.

The plasma half-life for circulating catecholamines reported in salmon in this study agrees with that reported in the rainbow trout by Nekvasil and Olson (1986) of less than 10 minutes for both adrenaline and noradrenaline. Ungell and Nilsson (1979) also reported a rapid disappearance of plasma catecholamines (under 10 minutes for adrenaline) in the Atlantic cod *Gadus morhua*. However, Mazeaud & Mazeaud (1973) found that in trout, the half-life of an injected dose of adrenaline or noradrenaline was 65.8 minutes and 163 minutes respectively. In all these studies, half-life was determined by injection of radioloabelled catecholamines, either intra-arterially, (Nekvasil and Olson, 1986; Ungell and Nilsson, 1979) or via intra-peritoneal injection (Mazeaud & Mazeaud 1973). Mazeaud & Mazeaud (1973) calculated the half-life based on metabolism and excretion of catecholamines.

Catecholamines released into the circulation are rapidly cleared by a combination of tissue accumulation/binding and metabolic degradation. In the atlantic cod, *Gadus morhua*, there is a bi-phasic disappearance of radiolabelled catecholamines from the plasma, with an initial fall followed by a slow decline (Ungell & Nilsson, 1979). This is also the case in other species studied such as the Goldfish (*Carassius auratus*) and the rainbow trout (*Salmon gairdneri*) (Busacker & Chavin, 1977; Nekvasil & Olson, 1986). The initial rapid phase predominantly reflects uptake into the tissues, while the slow phase

indicates subsequent release, catabolism and excretion (Ungell & Nilsson 1979). Therefore a determination of catecholamine half-life based solely on measurement of plasma disappearance alone does not take into account uptake of circulating adrenaline and noradrenaline by various tissues and the generation of various catabolites. This could account for the discrepancy between the reported half-lives for adrenaline and noradrenaline in the rainbow trout.

In the current study, it was possible to fit a single decay line to individual fish. This indicates that it is unlikely that there was any further substantial release of catecholamines into the circulation following surgery. This supports the idea that the catecholamine release is episodic in this species, which is apparent from studies on perfused cardinal veins to be reported in chapter 4. Following surgery, some fish struggled violently to escape confinement from the experimental boxes. This situation could be deemed as 'stressful'. Perry and Bernier (1999) have concluded that only severe stress will elevate plasma catecholamines. Their release does not appear to be a graded response along a continuum.

Catecholamine metabolism.

The removal of plasma catecholamines can occur either by excretion of the drug or uptake by organs, where the amines are broken down to the corresponding metabolites. There are two major enzymes involved in the breakdown of catecholamines; monoamine oxidase (MAO) and catechol-o-methyl-transferase (COMT). These produce deaminated and O-methylated catabolites respectively (Randall & Perry 1992). COMT metabolites are seen in the plasma before those attributed to MAO due to the fact COMT is a cytoplasmic enzyme whereas MAO is found in the mitochondria. A brief outline of catecholamine catabolism can be seen in Figure 2.17. In rainbow trout, it is unclear as to the relative importance of deamination versus O-methylation with regards to deactivation of biologically active catecholamines. Ungell & Nilsson (1979) found that in the cod, O-methylation was the pre-dominant pathway although to a lesser extent than determined in the rainbow trout (Mauzead & Mauzead, 1973). In contrast, Nekvasil & Olson (1986) found that deaminated catabolites were predominately found in rainbow trout suggesting a predominance of MAO initiated degradation. The authors state however that the major urinary catabolites in trout, MHPG and VMA (refer Figure 2.17) are actually formed from the enzymatic activity of both MAO and COMT and can therefore be regarded as both deaminated and O-methylated catabolites. If this is the case then pooling of these

metabolites with other deaminated products would tend to overestimate the role of deamination in the experimental animal (Nekvasil & Olson, 1986).

In the current study, measurements of tissue uptake and urinary excretion were not performed so COMT versus MAO pre-dominance cannot be reliably commented on. However, it is reasonable to assume that catecholamine degradation and catabolism in the salmon follows a similar trend as that seen in the rainbow trout.

There are several tissues in teleost fish that have been shown either directly, or indirectly to have significant MAO and COMT activity, including the gills, liver and kidney. Direct assay of the MAO enzyme from the tissues of rainbow trout indicated that the intestine exhibited greatest MAO activity, followed by the liver and brain (Edwards et al., 1986). The un-differentiated kidney displayed the least MAO activity although it had a high Km value (Edwards et al., 1986). Nekvasil & Olson (1986) found that on a per gram basis the spleen and kidney of the rainbow trout concentrated more radio-labelled adrenaline or noradrenaline than any other tissue studied. In the atlantic cod the greatest uptake was found in the posterior cardinal vein (chromaffin tissue), head kidney, kidney, heart and gill (Ungell, 1985).

There are two methods by which tissue can accumulate catecholamine from the plasma, neuronal (type 1) or extraneuronal (type 2). Neuronal uptake involves the absorption of catecholamines by adrenergic nerve terminals where the amines are either metabolised or packaged into secretory vesicles as neurotransmitters. Extraneural uptake refers to the accumulation by non-neural tissues for metabolic degradation (Randall & Perry, 1992). The significance of a particular organ to catecholamine metabolism is dependent on several factors at any one time. These include innervation density, blood flow, relative mass, endogenous catecholamine stores of the tissue and affinity of the degrading enzymes for catecholamines (Ungell, 1985, Randall & Perry, 1992). For example, although the liver in both trout and cod has been shown to accumulate only a small percentage of radiolabelled amines relative to the kidney, its mass may represent up to 4% of total body weight versus 0.1% for the head kidney (Ungell, 1985). Coupled with this is the fact that in rainbow trout, the liver has been shown to have a far higher MAO V_{max} (maximal reaction velocity) than the kidney (1.89 ± 0.25 and 0.40 ± 0.08 μmol/g/hr respectively). However, the Km of the kidney is significantly higher than that of the liver. This fact coupled with a low Vmax suggests that MAO present in the kidney is less efficient at deamination of catecholamines than other tissues (Edwards et al., 1986). The significant accumulation of catecholamines in the kidney could be due to passive diffusion

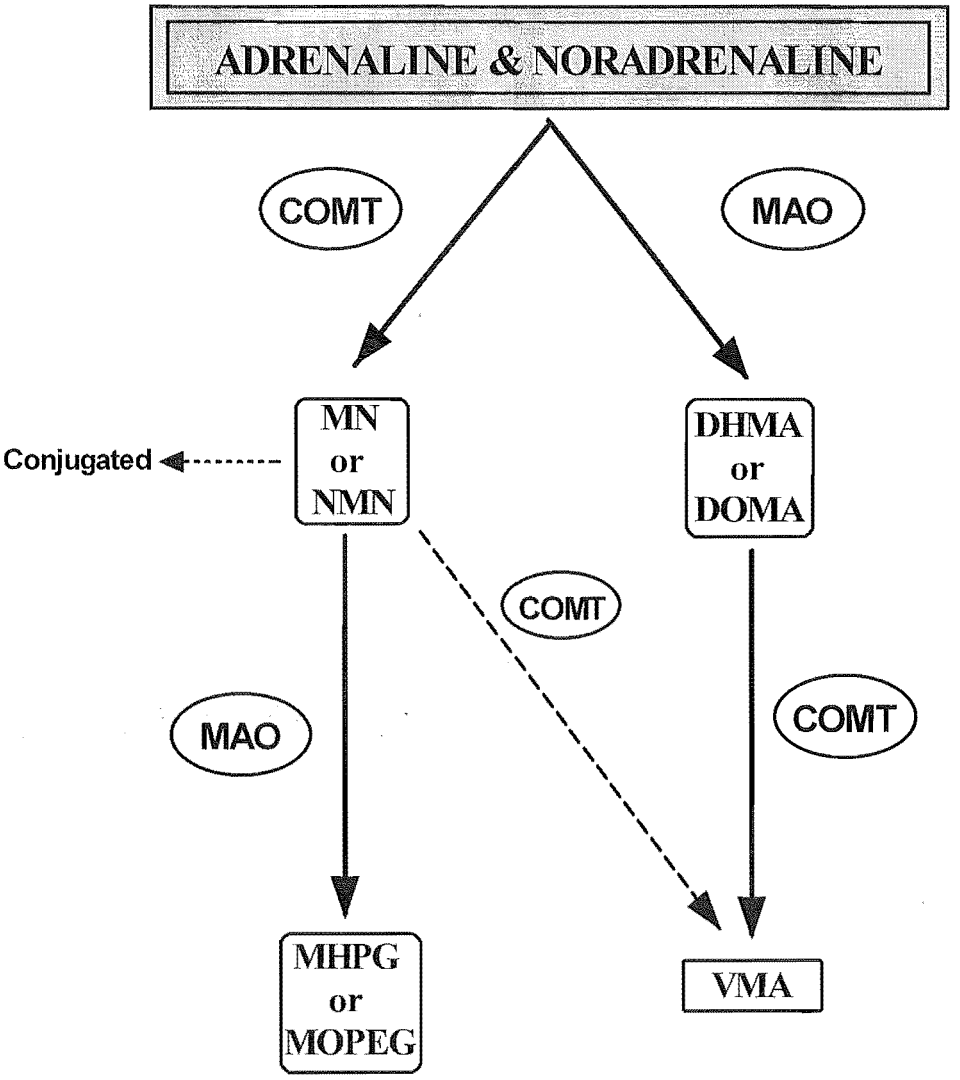


Figure 2.17. Diagram showing an outline of catecholamine metabolism in teleost fish. Catechol-O-transferase (COMT) breaks down adrenaline and noradrenaline to either MN (metanephrine) or normetanephrine (NMN) respectively. These products can then be conjugated and excreted, or O-methylated via MAO to MHPG or MOPEG (3-methoxy-4-hydroxyphenylglycol) or VMA (3-methoxy-4-hydroxymandelic acid). Alternately, monoamine oxidase (MAO) converts adrenaline and noradrenaline to DHMA or DOMA (3,4-dihydroxymandelic acid). These can then be converted to VMA via COMT before excretion. (modified from Nekvasil & Olson (1996)).

rather than a neuronal uptake by the chromaffin cells, which exhibit little or no specific neuronal uptake (Ungell, 1985).

Skeletal muscle may also represent a biological 'sink' for elevated catecholamine levels. In the atlantic cod and rainbow trout, skeletal muscle can account for up to 67% of total body mass and due to its large size, can accumulate over half the total amines absorbed by all tissues (Nekvasil & Olson, 1986), representing an important tissue for the deactivation of catecholamines. Uptake into vascular smooth muscle likely accounts for any active uptake of catecholamines in the skeletal muscle. There is also a component of passive diffusion into the muscle due to the low endogenous levels of adrenaline and noradrenaline (Ungell, 1985).

Possibly the most significant site of catecholamine excretion is at the gill. Due to the 'single circuit' circulation of the fish, the entire cardiac output perfuses the branchial tissues (Nekvasil & Olson, 1986) and the gills are the only organ whose microcirculation is in direct series with the systemic system. Therefore, the gill can be exposed to high concentrations of inflowing catecholamines and must have some mechanism of coping with these amines. Although excretion is not as rapid as direct tissue uptake it is important in lowering the total amine content in the whole animal (Nekvasil & Olson, 1986). The major function of the gill in regulation of plasma catecholamine levels is the deactivation of adrenaline and noradrenaline via removal from the plasma and/or metabolism to inactive products (Colletti & Olson, 1988). Studies on the perfused gill of the rainbow trout have shown they are highly efficient at extracting and metabolising plasma catecholamines, deactivating over 70% and 60% of a 10^{-6} noradrenaline and adrenaline bolus within 20 minutes, respectively (Colletti & Olson, 1988).

Extraction efficiency of the gill appears to be augmented in the face of increasing plasma catecholamine concentrations. During perfusion with 10^{-6}M catecholamines, the extraction of measurable amines was 75-80%, whereas during 10^{-8}M perfusion, extraction was around 65-70% (Colletti and Olson, 1988). The reasons suggested for this are either increased diffusive loss of catecholamines across the gill epithelium, inability of HPLC to detect unknown metabolites in the gill effluent or an enhanced extraction during a high catecholamine challenge. Although further studies will hopefully elucidate this possibility, it could be advantageous for the gill to extract catecholamines from the plasma in a somewhat dose dependent manner.

Given that in the present studies blood samples from snapper were obtained prior to the gill and salmon blood was sampled post-gill, it might be expected that snapper blood would contain higher concentrations of plasma catecholamines. This was not the case. It is

possible that snapper have a less well-defined catecholamine response to hypoxia than salmon and therefore simply release lower concentrations of catecholamines during hypoxia. This would be feasible given that snapper are highly unlikely to encounter hypoxic situations during their normal life cycle and might rely more heavily on behavioural rather than hormonal mechanisms to deal with reduced $P_{W_{O2}}$. However, it is also a possibility that adrenoceptors in snapper have a higher affinity for catecholamines than salmon. If this is the case, then it would take a lower concentration of catecholamines in snapper blood to elicit the same response as a higher concentration if catecholamines in salmon blood. Another possibility is that due to the extended time taken for surgery, plasma catecholamines may have begun to be eliminated by the gill (which was constantly irrigated) and/or absorbed by various tissues. Therefore the initial blood sample might not accurately reflect peak circulating plasma catecholamine concentrations.

In many teleost species studied, there is a significant difference in the way tissue accumulate noradrenaline versus adrenaline. Goldfish, cod and rainbow trout tissues all exhibit a preference for noradrenaline uptake over adrenaline accumulation (Busacker & Chavin, 1977; Ungell, 1985; Nekvasil & Olson, 1986). Ungell (1985) argues that the preferential accumulation of noradrenaline in the PCV and head kidney of the atlantic cod may be due to restriction of adrenaline uptake as the tissues already contain high concentrations of stored adrenaline. As adrenaline is the pre-dominant catecholamine in many teleost species (Randall and Perry, 1992) there may be several mechanisms acting to specifically remove noradrenaline from the circulation. If adrenaline is the most effective catecholamine in teleosts, specific uptake of noradrenaline will reduce competition for binding sites between the two amines. This would allow a maximal expression of the sympathetic response to adrenaline (Nekvasil and Olson, 1986). Alternatively, or in conjunction, noradrenaline may in fact be accumulated by some tissues in order to replenish catecholamine stores and serve as a substrate for the formation of further adrenaline. It is possible that this is the case in mammals, where noradrenaline is selectively removed from the circulation while adrenaline is not (Ginn & Vane, 1968, cited in Nekvasil & Olson, 1986).

In the present study, the half-life of noradrenaline in salmon plasma was longer than that calculated for adrenaline in the same experimental animals. In the cod, goldfish and rainbow trout, noradrenaline tissue accumulation was maximal by approximately 8 minutes post-injection of labelled amine (Ungell & Nilsson, 1979; Buschaker & Chavin,

1977; Ungell, 1985), and calculated plasma half-life was less than 10 minutes (Nekvasil & Olson, 1986). Although the initial blood sample for catecholamine determination in the study was taken at surgery, the following sample was taken when the fish began ventilating unaided. This was in excess of 10 minutes for some fish and this could mean that the initial rapid phase of noradrenaline disappearance from the plasma was not measured. However, 3 individuals showed plasma noradrenaline half-lives of less than 9 minutes, which is closer to other published values. The mean noradrenaline half-life value of approximately 11 minutes may represent the end of the rapid phase of disappearance and the start of the gradual phase, similar to that described by Ungell & Nilsson (1979).

Catecholamines are important circulating hormones that during times of acute stress and hypoxia are important in maintaining adequate tissue oxygenation in teleost fish. However, the high plasma concentrations that can be released need to be rapidly cleared before their advantageous actions are offset by their detrimental actions. This study, and those on the rainbow trout and Atlantic cod have shown that these species are highly efficient at lowering plasma catecholamine levels, even in the face of micro-molar concentrations. This is achieved through a combination of absorption, excretion and metabolism by body tissues. Tissue accumulation is dependent on several factors, and rapidly accumulating tissues probably receive a substantial portion of cardiac output, such as the gill. The relative proportion of adrenergic innervation is also likely to be of importance (Ungell, 1985). There appear to be subtle differences in the relative importance of individual tissues in catecholamine metabolism between species, along with the primary mechanism of metabolism. Regardless of these differences, it is plainly apparent that teleost fish have developed well regulated and effective mechanisms to adjust plasma catecholamine levels, thus allowing them to maintain optimal cardiovascular function during adverse conditions.

Chapter 3

Effects of hypoxia on *in situ* catecholamine release and the red blood cells of Chinook salmon.

Introduction

Recent studies have implicated the importance of arterial blood oxygen content in stimulating the release of catecholamines into the circulation. This release seems to correspond to a reduction of (Hb)-O_2 saturation of approximately 50% i.e. the P_{50} value (Perry and Reid, 1994; Perry and Bernier, 1999). This appears to be the case for several fish species including the rainbow trout and the American eel (Reid et al., 1998). Although in these species, catecholamine release occurs at markedly different PaO_2 values, these correspond with the specific P_{50} of the blood for each species. These results indicate that it is more likely to be a decrease in actual blood oxygen content than a fall in oxygen partial pressure that is the proximate stimulus for catecholamine release under hypoxaemic conditions (Perry and Bernier, 1999). However, several studies have put forward a strong correlation between blood lactate levels and catecholamine release (Boutilier et al., 1986; Aota et al., 1990). There is some contention as to whether these studies represent reality in terms of what is considered normal fish behavior (Reid et al., 1998). Classic 'stress' studies that aim to induce blood acidosis along with high catecholamine release often employ methods such as chasing fish to exhaustion or tail grabbing. These methods involve constant interaction with the fish, which in turn can cause severe stress responses to occur. Also, it is unlikely that fish in the wild would repeatedly swim to physical exhaustion. However an exception to this may occur during spawning where salmon are known to climb up dams that block their home rivers. It would seem disadvantageous for high lactate levels, a situation the fish would only rarely be exposed to, to be the main stimulus for catecholamine release. This idea is supported by a recent study indicating that blood acid-base status does not directly affect

catecholamine secretion or the blood oxygen content threshold for catecholamine release during hypoxia (Julio et al., 1998). It may however modulate the secretory process specifically in response to nicotinic receptor stimulation of chromaffin cells.

In teleost fish such as the salmonid family, it is generally accepted that nicotinic receptors are the dominant cholinoceptor present on chromaffin cells (Reid and Bernier, 1998). The nicotinic nature of cholinergic induced catecholamine secretion in teleost fish has been proven in several studies. The Atlantic cod, *Gadus morhua* releases catecholamines in *in situ* preparations in response to either electrical stimulation of the sympathetic nerves or application of acetylcholine (Nilsson et al., 1976; Montpetit and Perry, 1999; Perry and Bernier, 1999). This secretion can be inhibited or stopped by the addition of the ganglionic blocker hexamethonium, which inhibits nicotinic cholinoceptors (Reid and Bernier, 1998).

Nicotinic receptors are not the only receptor type found on chromaffin cells. Across vertebrate families, muscarinic receptors are found to be involved in stimulation of catecholamine secretion and knowledge of their role in catecholamine secretion in the trout is growing rapidly. Recent evidence has indicated that although muscarinic receptors may not represent a proximate pathway for catecholamine release, they may enhance nicotinic stimulated catecholamine release, particularly during acidosis (Julio et al., 1998).

In the present study, experiments were carried out to investigate the effects of hypoxia and anaesthetic exposure on the release of catecholamines by the chromaffin tissue of the posterior cardinal vein of the Chinook salmon. During anaesthesia, tissues might be affected directly by the anaesthetic (AQUI-S in this case), or indirectly by the low oxygen partial pressures due to reduced ventilation. Both of these influences were investigated, as was the direct effect of hypoxia on red blood cells *in vitro*.

Methods and Materials

Series 3.1 Direct effects of hypoxia and AQUI-S anaesthetic on catecholamine release from the posterior cardinal vein of salmon.

Experiment 3.1.a. Direct effects of hypoxia on the chromaffin tissue of the Chinook salmon.

Chinook salmon (*Oncorhynchus tshawytscha*, mean weight 1390.2 ± 115.8g, ± 1SEM), range 989.1–1654.0g, n=6) were transported from Isaacs Salmon farm (McLeans Island, Christchurch, New Zealand) and held in a large darkened fibreglass tank (1.5 x 1.5 x 1.5 m) supplied with constantly flowing fresh artesian water. Water temperature ranged from 12-13°C throughout the experimental period and fish were maintained on a 12:12 hour light:dark regime.

When required, fish were removed from the holding tank and killed with a sharp blow to the head. 1ml of heparinized freshwater teleost saline was injected via the caudal vein and allowed to circulate for 1 minute. Fish were then placed ventral side up in an operating sling and a midline incision made from the anal fins to the tip of the lower jaw. The floor of the pericardium was carefully dissected away and heart exposed. A small transverse incision was made in the bulbus arteriosus close to the start of the ventral aorta. The tip of a 10cm length of polythene cannula (OD 1.00 x ID 0.50mm, Portex Tubing) was then inserted approximately 1cm into the bulbus. Care was taken not to disrupt the heartbeat or move the cannula into the ventricle (Figure 3.1). Surgical thread was used to secure the cannula by tying a ligature around the entire bulbus arteriosus at the point where the cannula was inserted. This allowed the cardiac output to exit *via* the cannula. The free end of the cannula was then attached to a variable height clamp stand. This provided the means to adjust afterload on the heart (Farrell et al., 1986). The outflow from the cannula was set between 5-10cm above the level of the heart depending on the force of the heartbeat.

The tail posterior to the anal fins was then removed and a 10cm long metal cannula (OD approx. 1.0 x ID approx. 1.0mm) with a flexible polythene tip, inserted into the posterior cardinal vein (PCV) of the kidney (Figure 3.1). The cannula was secured with a ligature running through the body musculature, around the spine and encompassing

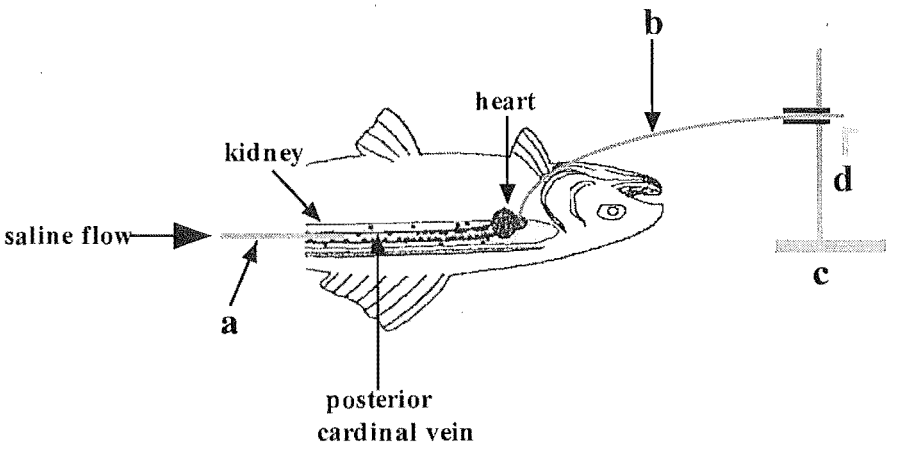


Figure 3.1 Diagram showing the cannulation of posterior cardinal vein (PCV) and heart of Chinook salmon for experiment 3.1a and 3.1b. The PCV was perfused with saline or anaesthetic through a cannula (**a**). Perfusate was collected via a cannula inserted in the bulbus arteriosus (**b**) whose tip was secured to a variable height clamp stand (**c**). Samples were collected over 30 second periods in micro centrifuge (eppendorf) tubes (**d**). See text for description of electrode placement and further details.

the kidney tissue. The preparation was then perfused with aerated freshwater teleost saline of the same composition as that used in Experiment 2.1, delivered from either one of two 100ml reservoirs. Once perfusion flow was established and the heartbeat regular, fine platinum electrodes (approx. 1mm in diameter, 35.5mm length) were inserted into the muscle under the peritoneum, along either side of the body wall running parallel with and approximately 2mm above the kidney tissue. The electrodes were then connected to a current stimulator (Danish Myo Technology Current Stimulator, Model CS200) set to deliver a single, 2-second pulse of 32Hz with a current of 120mA. This setting was chosen after several experiments were performed to find the threshold current required by this stimulator to cause significant catecholamine release as determined by HPLC analysis.

Flow was achieved by using a constant pressure head system with an overflow valve. Saline was gassed using a Wösthoff gas pump, (Wösthoff, Type 1 M 100/a, Bochum, Germany) which allowed a specific CO_2/O_2 gas mixture to be 'dialled' up and then delivered through an air-stone to the saline. Saline was maintained at a constant temperature of 11°C and the PO_2 monitored by a flow through O_2 electrode (OM-4 Oxygen meter, Microelectrodes Inc. Bedford, New Hampshire, USA) connected in-line with the in-flow cannula, approximately 10cm before entering the fish. The preparation was then left to perfuse with normoxic saline (gassed with a 19% O_2/0.5% CO_2 mix in air) for 20 minutes until red blood cells were no longer apparent in the out flowing perfusate

and the heartbeat was strong and regular. It was also assumed that this would be long enough for any catecholamines released from the fish during death to be cleared from the preparation. After this time, the preparation was perfused with normoxic saline for a further 15 minutes. This was deemed the control period prior to experimentation. In the last 6 minutes of the control period (starting the 9th minute), perfusate samples were collected from the outflow cannula every 3 minutes. Samples were collected in pre-weighed labelled Eppendorf tubes for a 30 second period. At the end of sampling the tube was weighed again on a balance (Mettler PC440) and the flow rate determined per minute. The sample was then frozen and held for no longer than 1week at –80°C prior to HPLC catecholamine analysis. Catecholamine analysis protocol was identical to that described for Series 2.1 and 2.2 in the previous chapter.

At the end of the control period, flow was changed via a tap to saline gassed to give a final concentration of 4.2% dissolved oxygen (20% of the normoxic saline oxygen content). During this and consecutive treatments, samples were collected every 3 minutes of the 15 minute treatment period. Collecting samples 3 minutes into the treatment allowed the chromaffin tissue to be exposed to hypoxic saline prior to any sampling. Samples were handled and processed as described above.

Following exposure to 4.2% dissolved oxygen saline the preparation was perfused for 15 minutes with 2.1% dissolved oxygen saline, 1.05% dissolved oxygen saline and then exposed to anoxic saline (0% dissolved oxygen). These concentrations represent 20%, 10% 5% and 0% of oxygen content in normoxic saline. Finally, the preparation was returned to normoxic (aerated) saline for a final 15 minutes. Samples were taken throughout all treatments and the PO_2 monitored constantly to ensure saline was hypoxic. Although in general the heart preparation lasted throughout the experiment, on exposure to very low oxygen concentrations, the heart rate slowed. When this occurred, the after load was decreased until a regular heart rate was achieved. This was usually about 8-10 beats per minute.

After the final normoxic period, the preparation was stimulated with an electrical current from the current stimulator set at 32 Hz, 10ms duration 120mA. The total duration of stimulation was 2 seconds. Following stimulation, perfusate samples were collected as above, each minute for the following 5 minutes. Direct electrical stimulation of the chromaffin tissue allowed the physical ability of the chromaffin cells to release catecholamines in any given preparation, to be assessed.

Samples were analysed for the presence of catecholamines by HPLC analysis as previously described. Total catecholamine concentration was multiplied by the flow rate for any given sample to give catecholamine secretion rate in picomoles per minute. It is possible that heart tissue might bind and metabolise some catecholamines produced by the chromaffin tissue, which would lead to an underestimation of release rate.

Data were analysed using one-way repeated measures analysis of variance (ANOVA) with Bonferroni post-test analysis. This allowed comparison of data points between groups. In all cases $P \leq 0.05$ was used to indicate a significant difference.

Experiment 3.1.b. Effects of AQUI-S on evoked catecholamine release from the chromaffin tissue of the Chinook salmon.

Chinook salmon (*Oncorhynchus tshawytscha*, mean weight 877.60 ± 118.64g, (±1sem) range 368.4-1353.0g, n=7) were transported from Isaacs Salmon farm (McLeans Island, Christchurch, New Zealand) and held at the University of Canterbury as previously described.

When required, fish were removed from the holding tank and killed with a sharp blow to the head. 1ml of heparinized freshwater teleost saline was injected via the caudal vein and allowed to circulate for 1 minute. The PCV was then cannulated, perfused and platinum electrodes inserted along the muscle lining the body cavity using the same methods as previously described in Experiment 3.1.a. However, the experiment protocol was modified in the following ways; throughout the experiment the preparation was perfused with aerated saline and, following the initial control period, the preparation was electrically stimulated (using the same settings as described in Experiment 3.1.a) and perfusate samples collected over the subsequent 15 minutes. The preparation was then stimulated again and further samples collected. After this second stimulation, the preparation was perfused with saline containing AQUI-S anaesthetic at a final concentration of 200ppm. There was a 10-minute period to allow the anaesthetic to saturate the PCV. Then a further 2 bouts of stimulation and sample collection were carried out. The preparation was then perfused with anaesthetic free saline for a final 2 stimulations and samples collected. In total the preparation was electrically stimulated 6 times.

Perfusate samples were collected, stored and analysed in the same way as described in Experiment 3.1.a.

Series 3.2 Direct effects of hypoxia on teleost red blood cells *in vitro*.

Experiment 3.2 The direct effect of hypoxia on the red blood cells of Chinook salmon *in vitro*.

Chinook salmon (*Oncorhynchus tshawytscha*, mean weight 729.61 ± 158.53g, (±1SEM), range 278.7-1135.0g, n=7) were transported from Isaacs Salmon farm (McLeans Island, Christchurch, New Zealand) and held in a darkened fibreglass tank (1.5 x 1.5 x 1.5 m) supplied with constantly flowing fresh artesian water. Water temperature ranged from 12-13°C throughout the experimental period and fish were maintained on a 12:12 hour light:dark regime.

When required, fish were removed from the holding tank and killed with a sharp blow to the head. 1ml of heparinized freshwater teleost saline was injected via the caudal vein and allowed to circulate for 1 minute. As much whole blood as possible (usually 8-9ml) was then withdrawn *via* caudal puncture into a heparinized syringe and injected into a tonometer flask. The blood was gassed with humidified air (19% oxygen, 0.5% carbon dioxide, Wösthoff, type 1 M 100/a gas pump Bochum, Germany) in a spinning tonometer (Instrumentation Laboratory 237) for 60 minutes. In the final 40 minutes of normoxia, blood was sub-sampled every 10 minutes and pH measured using a pH/Blood gas analyser (Instrumentation Laboratories, Micro 13 and Temp controller 323). Approx. 200µl of whole blood was taken for each sub-sample. Haematocrit (Hct) and MCHC were also measured using methods previously described. After pH measurement, the blood was returned to the tonometer flask to minimize blood volume changes. After this control period, blood was gassed with a 90% N_2, 10% of 5% CO_2 in N_2 mix for 60 minutes to simulate hypoxia. During the final 40 minutes of anoxia, blood sub-sampling continued as described above.

Finally, the blood was returned to normoxic conditions for an additional 60 minutes, with sub-sampling for the final half hour.

Data was analysed using a Students 't' test to determine differences between variables during normoxia and hypoxia.

Unless otherwise stated, significance levels were set at $P < 0.05$.

Results

Series 3.1 Direct effects of hypoxia and AQUI-S anaesthetic on catecholamine release from the posterior cardinal vein of salmon

Experiment 3.1.a. Direct effects of hypoxia on the chromaffin tissue of the Chinook salmon.

Catecholamine secretion did not increase in response to a step-wise decrease in oxygen tension of the perfusion saline. The mean adrenaline release during each hypoxic treatment is shown in Figure 3.2. Between replicates, total adrenaline release calculated over an entire hypoxic exposure ranged from a low of 3.84pMmin^{-1} for one preparation, to a high of 75.23pMmin^{-1} for another. Only one preparation released detectable, albeit sporadic concentrations of noradrenaline, even during electrical stimulation. Catecholamine release from the preparations appeared to be sporadic and transient. Preparations either a) released low adrenaline concentrations throughout hypoxia followed by high concentrations during electrical stimulation; b) released high adrenaline concentrations at the start of perfusion with concentrations falling throughout the experiment with no significant release following electrical stimulation; or c) released sporadic 'spikes' of adrenaline throughout the experiment with no significant release following electrical stimulation. Examples of individual experiments showing the diversity of adrenaline release patterns between preparations can be seen in Figure 3.3a-c.

Electrical stimulation generally elicited a highly significant ($P \leq 0.0001$, n=6) adrenaline release from the posterior cardinal vein preparation (Figure 3.2) when compared to release at any other time during the experiment.

Table 3.1 shows means values for partial pressure of oxygen (PO_2, %), flow rate (per minute) adrenaline secretion rate (pMmin^{-1}) and absolute adrenaline concentration in nM during each experimental treatment. Note that the flow rate during hypoxia decreased markedly but was restored during normoxia. As collection depended on the heart pumping

Saline [O_2]	PO_2 meter reading (%)	Flow Rate (ml min^{-1})	Adrenaline (pM min^{-1})	[Adrenaline] (nM)
Normoxia 1	19.0 ± 0.6	1.2 ± 0.2	51.7 ± 8.7	43.8 ± 7.5
20% Normoxia	5.0 ± 0.3	0.7 ± 0.1	43.1 ± 13.6	53.0 ± 13.1
10% Normoxia	3.0 ± 0.1	0.5 ± 0.1	24.5 ± 6.5	41.9 ± 9.7
5% Normoxia	1.2 ± 0.3	0.8 ± 0.1	57.7 ± 24.1	45.2 ± 12.2
0% Normoxia	0.4 ± 0.3	0.6 ± 0.1	25.1 ± 7.3	33.7 ± 6.0
Normoxia 2	21.0 ± 0.6	1.3 ± 0.2	31.0 ± 6.7	28.9 ± 8.0
Stimulation	21.0 ± 0.6	1.2 ± 0.2	166.3 ± 39.9	120.6 ± 29.7

Table 3.1 Table showing mean oxygen concentration (taken as % O_2 from flow through oxygen meter) of inflowing saline, flow rate out of the preparation (ml min^{-1}), total adrenaline secretion rate (pM min^{-1}) and absolute adrenaline concentration (nM) from the perfused PCV during normoxia, subsequent hypoxia and electrical stimulation. Saline oxygen concentration is represented as a percentage of normal oxygen concentration (19% O_2) The preparation was perfused with normoxic (aerated) saline during electrical stimulation.

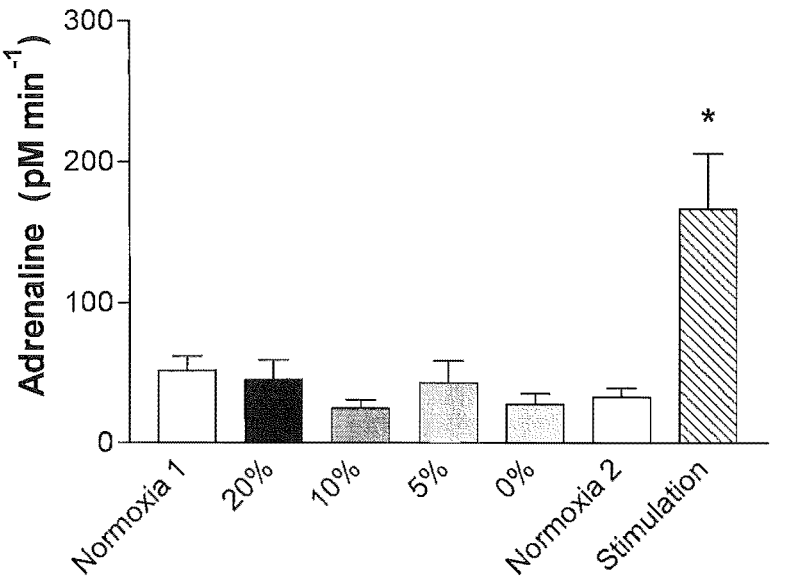


Figure 3.2 Mean Adrenaline secretion rate (pMmin^{-1}) from the perfused PCV of the Chinook salmon during normoxia, progressive hypoxia and following electrical stimulation. There was no significant increase in adrenalin secretion in response to hypoxia. * denotes a significant difference from all other times ($P \leq 0.001$, n=6)

perfusate into the bulbus arteriosus and outlet cannula, concentrations of adrenaline measured during hypoxic periods did not fall as much as Figure 3.2 suggests.

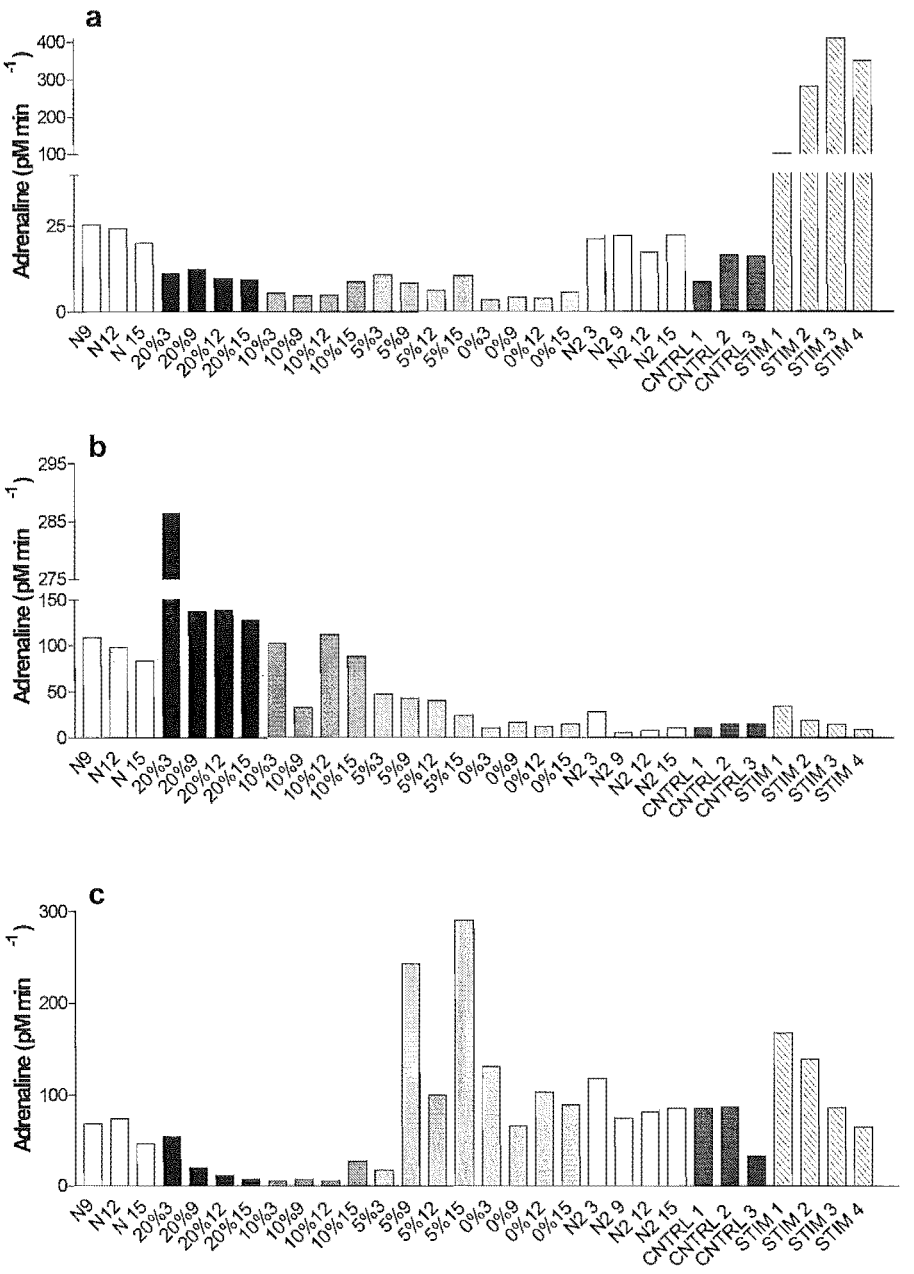


Figure 3.3a-c. Adrenaline secretion patterns of 3 individual experiments from Experiment 3.1a. Preparations either a) released low adrenaline concentrations throughout hypoxia followed by high concentrations during electrical stimulation; b) released high adrenaline concentrations at the start of perfusion which fell throughout the experiment with no significant release following electrical stimulation; or c) released sporadic 'spikes' of adrenaline throughout the experiment with no significant release following electrical stimulation.

Experiment 3.1.b Direct effects of AQUI-S on the chromaffin tissue of the Chinook salmon.

Total catecholamine release varied from preparation to preparation, but within experiments stimulation consistently elicited catecholamine secretion. Mean values for adrenaline and noradrenaline secretion following each stimulation are shown in Figures 3.4 a & b and Table 3.2. Absolute values for adrenaline and noradrenaline release (nM) and flow rate data are also given in Table 3.2. Catecholamine release tended to decline over time, although secretion following the final stimulation (stim 6) was significantly higher ($P \leq 0.05$, n=6) than at all other times, excluding the first stimulation (stim 1).

Only one preparation consistently released noradrenaline throughout the experiment. All other preparations appeared to release noradrenaline at random intervals. However, more samples contained noradrenaline during AQUI-S exposure than at any other time, although the total concentration was not significantly different than any other time (Table 3.2). As with adrenaline, noradrenaline concentrations were significantly higher ($P \leq 0.05$, n=6) following the final electrical stimulation (stim 6) than at any other time, except following the first stimulation (stim 1).

During HPLC analysis, it was noted that several samples from 6 different experiments contained an unidentified peak, occurring consistently at 8.9 minutes on the HPLC chromatogram. The peak was only found in the two samples taken immediately prior to anaesthetic exposure (i.e. the last 2 samples of stimulation 2) and all samples taken in the presence of AQUI-S. It was not found in the samples taken during perfusion with normal (anaesthetic-free) saline. Given that the experiments were analysed several months apart with new mobile phase and other chemicals used in every analysis, it is unlikely this peak is a type of contamination. It is also unlikely to be a contaminant from the HPLC column as many other analyses were performed using the same column, which did not show this peak. Mean peak areas for this unidentified peak are shown in Figure 3.5, as concentration in picomoles min^{-1} cannot be calculated.

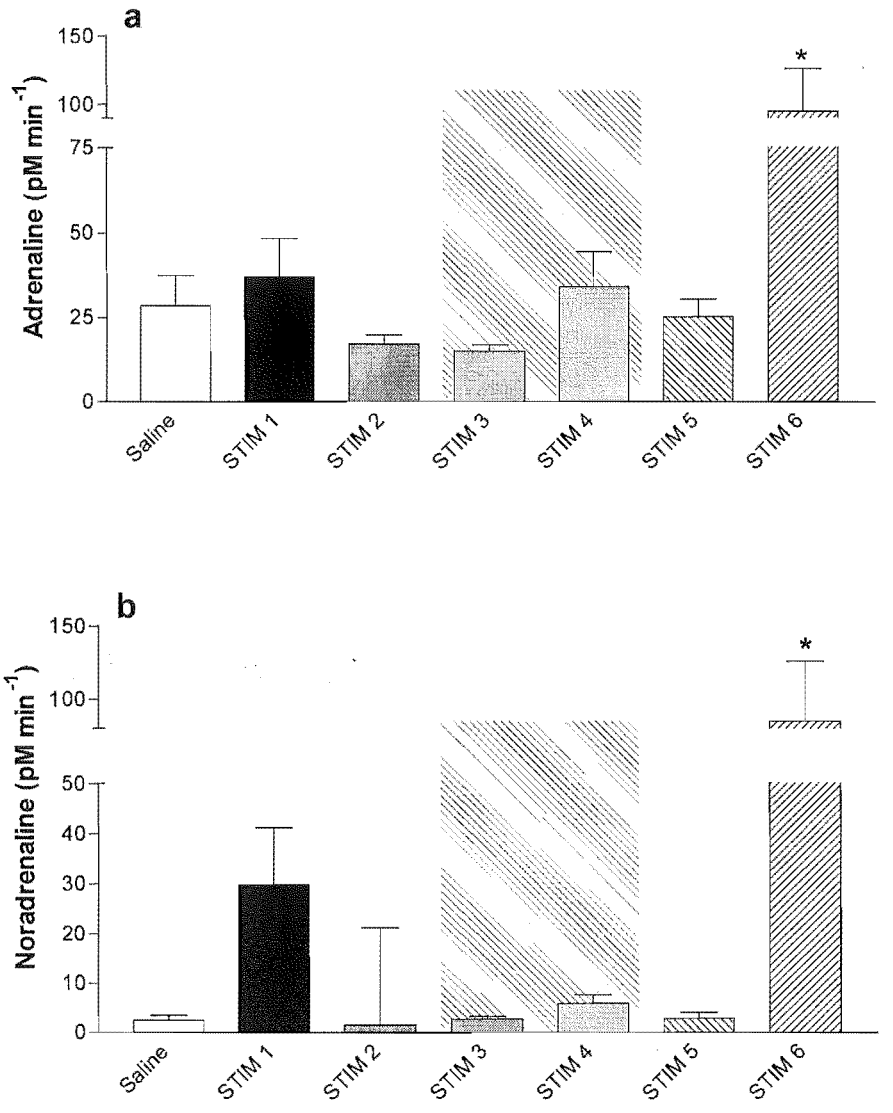


Figure 3.4 a&b. Mean adrenaline and noradrenaline secretion following electrical stimulation of the PCV in Chinook salmon. Saline is the control period prior to any electrical stimulation. Shading indicates stimulation in the presence of 200ppm AQUI-S anaesthetic. 'STIM' represents a 15 minute period following electrical stimulation. See text for further details.
* denotes a significant difference in catecholamine secretion compared to all other times except STIM 1.

	Flow Rate (ml min^{-1})	[Noradrenaline] (nM)	Noradrenaline (pM min^{-1})	[Adrenaline] (nM)	Adrenaline (pM min^{-1})
Saline	2.4 ± 0.3	16.3 ± 9.6	2.5 ± 1.0	29.5 ± 10.0	28.6 ± 9.0
STIM 1	2.8 ± 0.2	28.2 ± 15.7	29.8 ± 11.4	24.5 ± 5.1	37.1 ± 11.3
STIM 2	1.9 ± 0.2	7.3 ± 2.4	1.5 ± 19.6	16.0 ± 2.0	17.1 ± 2.7
STIM 3	0.9 ± 0.1	14.2 ± 5.1	2.7 ± 0.6	28.8 ± 3.4	15.1 ± 2.0
STIM 4	1.0 ± 0.1	17.2 ± 5.2	5.9 ± 1.7	43.3 ± 8.0	34.1 ± 10.4
STIM 5	1.4 ± 0.1	3.0 ± 1.5	2.8 ± 1.2	24.2 ± 3.8	25.2 ± 8.2
STIM 6	2.2 ± 0.3	42.3 ± 21.9	84.7 ± 41.0	43.3 ± 15.6	94.3 ± 31.5

Table 3.2. Table showing mean flow rate (ml min^{-1}), absolute noradrenaline and adrenaline concentrations (nM) and mean noradrenaline and adrenaline secretion rate (pM min^{-1}) from the perfused PCV of Chinook salmon following field stimulation (STIM 1-6). Grey shading indicates time when preparation was perfused with 200ppm AQUI-S.

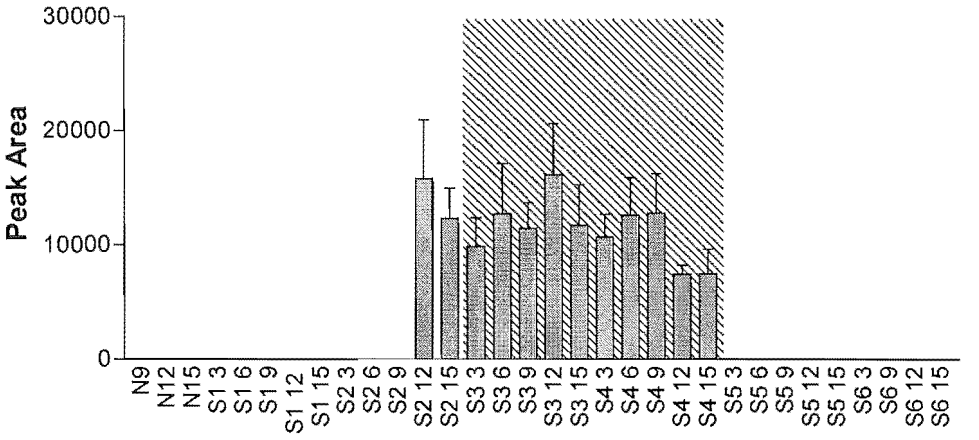


Figure 3.5 Graph of HPLC chromatogram peak areas of unknown substance detected during catecholamine analysis of perfusate samples from Experiment 3.1b. Shading indicates samples taken during perfusion with 200ppm AQUI-S. This unknown peak was not found at any other time during the experiment and was detected in 6 of 7 experiments.

Series 3.2 Direct effects of hypoxia on teleost red blood cells *in vitro*.

Experiment 3.2 The direct effect of hypoxia on red blood cells of the Chinook salmon *in vitro*.

The mean values for haematocrit, blood pH, and mean cell haemoglobin prior to hypoxic exposure were 37.28 ± 1.6%, 7.58 ± 0.05, and 23.95 ± 0.89g.dL^{-1} respectively. Graphs showing mean values for all three variables during normoxia and hypoxia are shown in Figure 3.6 a-c.

All variables showed significant yet reversible changes during hypoxia, with haematocrit and blood pH rising above normoxia values ($P \leq 0.0001$, n=18) while MCHC fell ($P \leq 0.01$, n=12). On return to normoxia, Hct and MCHC returned to values very close to pre-hypoxia. Mean pH fell slightly below pre-hypoxia values to 7.50 ± 0.06 (Figure 3.6 a-c).

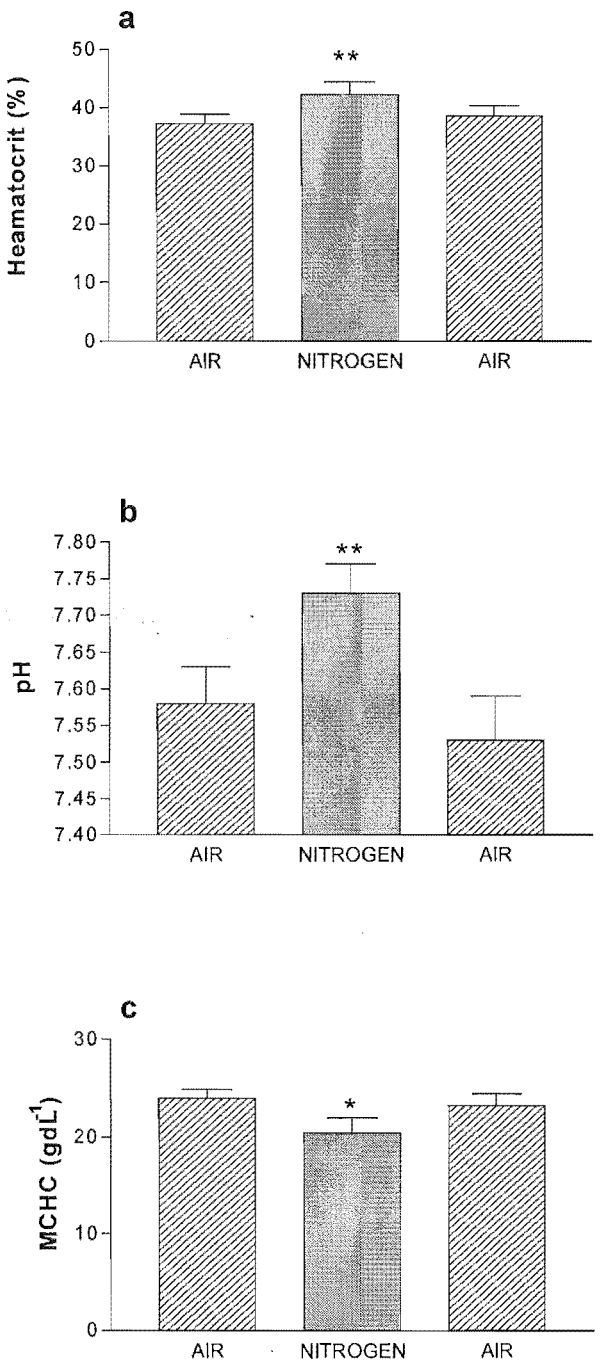


Figure 3.6 a-c. Mean values of Hct (%), pH_e and MCHC ($g.dL^{-1}$) for Chinook blood *in vitro* prior to, during and following hypoxic exposure. ** denotes a significant difference from pre-hypoxia values ($P \leq 0.0001$). * denotes a significant difference from pre-hypoxia values ($P \leq 0.05$).

Discussion

PCV Perfusions.

The perfused posterior cardinal vein preparation used in this study was based on well established methodology used by other investigators (Nilsson et al., 1976; Reid et al., 1996; Julio et al., 1996; Montpetit and Perry, 1999) and basal secretion rates for adrenaline were similar to other published studies (Julio et al., 1996, Perry et al., 2000). The lack of any increase in adrenaline release during exposure to hypoxia in the present study is unlikely to reflect an inability of the chromaffin cells to release adrenaline in this preparation. This is evidenced by the fact that subsequent electrical stimulation elicited a significant increase in adrenaline secretion rates compared to all other times throughout the experiment (Figure 3.2). However, the mean adrenaline secretion rate in response to electrical stimulation (166.33 ± 39.91 pM min^{-1}) was lower than in other studies (~ 800 pM min^{-1} Perry et al., 2000; ~ 300 pM min^{-1} Montpetit and Perry, 1999). The main difference between this and other studies is the type of electrical stimulus used. While other investigators have used electrical stimulation with a set voltage, usually 60–80V, (Montpetit and Perry, 1999; Perry et al., 2000; Montpetit and Perry, 2000; Montpetit and Perry, 2002) electrical stimulation using a constant current was chosen for the experiments presented in this study. However, there is variation between many studies employing nerve stimulation techniques: 32Hz, 10ms duration, 120mA current (Chinook salmon, present study), 20Hz, 1ms duration, 60V, (Rainbow trout, Montpetit and Perry, 1999; Perry et al., 2000; Montpetit and Perry, 2000;) 20Hz, 30s duration (Atlantic cod, Nilsson et al., 1976), 6Hz, 1ms duration, 8V (Eel, Abele et al., 1998). Also, the siting of electrodes varies. Nilsson et al., (1976) stimulated the nerve directly using hook electrodes, Abele et al., (1998) stimulated the nerve root of the hindbrain, and Perry and colleagues used field stimulation in their studies with rainbow trout.

The perceived advantage of using an electrical stimulator set to give a constant current rather than voltage is that the current stimulator takes into account variables such as the resistance of the tissue, distance between the electrodes and other factors, thereby adjusting the voltage accordingly to deliver a consistent stimulus to the tissue. Theoretically, this provides more consistent results from one preparation to the other. Setting a constant voltage through the tissue does not take into account these factors and may in fact be delivering a variable current to nerves and excitable cells from one preparation to the next.

Given that in the current study, adrenaline secretion rates following electrical stimulation were significantly higher than at any other time during the experiment, in these preparations at least, the stimulation protocol was valid in eliciting neuronally stimulated adrenaline release. That there was no significant noradrenaline release upon electrical stimulation is surprising. It is generally accepted that adrenaline is the predominant catecholamine in teleost fish (Randall and Perry, 1992). Many studies using either hypoxia or electrical stimulation *in situ* to elicit catecholamine release present data as the total rate of catecholamine secretion, combining both adrenaline and noradrenaline secretion rates (Perry et al., 2000; Montpetit and Perry 2002) or as values normalized to 100% (Abele et al., 1998). This makes it difficult to ascertain values for secretion rates of the individual catecholamines. However, of those studies that do differentiate between the two catecholamines, values for noradrenaline are usually at least 3-4 times lower than for adrenaline (Julio et al., 1998; Montpetit and Perry 2000). Differences in noradrenaline adrenaline secretion ratios may be related to differences in the innervation of adrenaline secreting cells versus noradrenaline secreting cells.

The effects of varying degrees of hypoxia on catecholamine secretion in teleost fish have been well documented (Aota et al., 1990; Fiévet et al., 1990; Gamperl, et al., 1994a; Julio, et al., 1998; Randall and Perry, 1992; Reid, et al., 1998). It is generally accepted that in most teleost fishes, catecholamine release occurs during extreme stress, when blood oxygen saturation falls below a critical value closely corresponding to the P_{50} of haemoglobin (50% blood oxygen saturation) (Randall and Perry, 1992; Perry and Bernier 1999). The predominant mechanism of catecholamine release from salmonid (and other teleost species) chromaffin cells is via increased neuronal stimulation by pre-ganglionic sympathetic nerve fibres innervating chromaffin cell cholinergic receptors (Reid et al., 1998; Montpetit and Perry, 1999). Acetylcholine release from these fibres stimulates nicotinic receptors to elicit secretion from the chromaffin cells.

Given that drastic reductions in arterial blood oxygen saturation are seen to occur prior to catecholamine secretion, is reasonable to assume that venous PO_2 and O_2 content must also be greatly reduced at this time (Perry, et al., 2000). There may also be other changes in blood chemistry, such as a developing state of hypercapnia resulting in changes in blood pH (Julio et al., 1998). Therefore it is likely that the interstitial fluid bathing the chromaffin cells of the posterior cardinal vein will be hypoxic/hypercapnic prior to catecholamine secretion. With this in mind, the aim of the experiments presented in this

chapter was to determine whether the chromaffin cells could release catecholamines in direct response to hypoxia, *in situ*.

It has previously been shown in the Atlantic cod (*Gadus morhua*) that perfusion of the chromaffin cells of the head kidney with hypoxic saline containing red blood cells causes a significant increase in basal catecholamine secretion (Perry et al., 1991) and based on these results it was suggested that there exists a local, direct stimulatory effect of blood hypoxemia on adrenaline release from teleost chromaffin tissue. This appears not to be the case however in salmon (present study) or in trout. Perry et al., (2000) investigated the effects of hypoxia on chemically and neuronally induced catecholamine release in the rainbow trout (*Oncorhynchus mykiss*) both *in vivo*, using chronically cannulated fish and *in situ* using perfused PCV preparations. As with the results presented here, Perry et al., (2000) found that perfusion with either hypoxic saline or hypoxic blood caused no significant increase in spontaneous catecholamine secretion rates from the perfused trout PCV *in situ*. However, using field stimulation techniques, it was found that neuronally evoked catecholamine secretion was decreased by 50% during perfusion with hypoxic saline *in situ*. This appears to by due to a direct inhibitory effect on the cholinergic receptors and indeed, in bovine chromaffin cells it has be shown that hypoxia reduces the ligand-binding affinity of nicotinic receptors (Lee et al., 1995; cited in Perry et al., 2000).

In contrast, *in vivo* experiments indicated an enhanced effect of hypoxia on nicotinic stimulated catecholamine release with a dose dependant elevation of plasma catecholamines in response to intra-arterial injections of nicotine (Perry et al., 2000).

Taking into account the *in situ* (present study, Perry et al., 2000) and *in vivo* (Perry et al., 2000) results of hypoxia on basal and neuronally evoked catecholamine secretion, it appears unlikely that there is a simple relationship between catecholamine release and blood PO_2 at the level of the chromaffin cells, at least in salmonids. Given that *in vivo* it has been clearly demonstrated that exposure to hypoxia causes significant increases in circulating plasma catecholamines (Wendelaar Bonga, 1997; Reid et al., 1998; Perry and Bernier, 1999) the results of *in situ* investigations imply the role of a functional higher control centre/s for release in the whole animal (Perry et al., 2000; Lapner and Perry, 2001). A possible candidate for this higher control centre may be O_2 chemoreceptors located on the gill arches of teleost fish, which are thought to be analogous to the O_2 sensing carotid body in mammals. These chemoreceptors are either externally oriented to monitor PO_2 of water or internally oriented to monitor changes in PO_2 of arterial blood (Reid and Perry, 2003). In rainbow trout, administration of cyanide

(an O_2 chemoreceptor stimulant) either to inspired water or injected into the gill circulation, caused a significant increase in plasma catecholamines. This effect was greatly enhanced during hypoxia, but inhibited during normoxia or hyperoxia (Reid and Perry, 2003). Removal of the first gill arch abolished catecholamine release in response to cyanide in inspired water but not to intra-arterial injection (Reid and Perry, 2003). *In situ* perfusion of PCV preparations illustrated a lack of any direct effect of cyanide on the chromaffin tissue. The authors conclude that O_2 chemoreceptors located on the gills initiate the reflex leading to release of catecholamines from the chromaffin tissue of trout.

In cod, bilateral sectioning of the glossopharyngeal and pretrematic ramus of the vagus innervating the first pair of gill arches did not impair the ability to respond to hypoxia (Fritsche and Nilsson, 1989). The authors concluded that the cod has 2 distinct mechanisms of responses to environmental hypoxia. The first is mediated by O_2 receptors on the gills innervated by the sectioned nerves, while the second involves O_2 sensors located elsewhere which trigger a rapid response to hypoxia (Fritsche and Nilsson, 1989). It is possible the gill arch O_2 receptors described in the trout by Reid and Perry (2003) are the same as those identified in the cod.

Considering the above results in trout and cod, it appears most likely that during hypoxia, the internally oriented O_2 chemoreceptors are the dominant mediators of catecholamine release in the rainbow trout (Reid and Perry, 2003). Given the similar results between studies in rainbow trout (Perry et al., 2000) and the present study, it is possible that this pathway also plays a key role in catecholamine secretion on exposure to environmental hypoxia in Chinook salmon and may explain the lack of catecholamine release seen in the PCV perfusion experiments.

As with salmon and rainbow trout, there does not appear to be a simple relationship between PO_2 of the fluid bathing the chromaffin cells and catecholamine release in hagfish (Perry et al., 1993, cited in Bernier and Perry 1996). However, recent studies on hagfish, eels and trout have identified various non-cholinergic neurotransmitters and/or neuromodulators, co-released with acetylcholine during neuronal stimulation, that elicit catecholamine release (Reid et al., 1996; Bernier and Perry, 1996; Abele et al., 1998; Bernier et al., 1999; Montpetit and Perry, 1999; Montpetit and Perry, 2000; Lapner and Perry, 2001). Some of these modulators include vasoactive intestinal polypeptide (VIP), adrenocorticotropic hormone (ACTH), pituitary adenylate cyclase activating polypeptide (PACAP) serotonin and angiotensin II (Ang II). Of these, ACTH and Ang II appear to be the most potent secretagogues of catecholamines with most studies investigating the role of Ang II due to the identification of specific Ang II

receptors on trout chromaffin cells (Lapner et al., 2000). However, their actions appear to be genus/taxon specific. For example, while ACTH and Ang II have both been shown to stimulate catecholamine secretion in the rainbow trout (Reid and Perry 1996; Bernier and Perry 1999; Lapner and Perry 2001) Ang II had no effect on secretion in the Atlantic hagfish, *Myxine glutinosa* (Bernier and Perry, 1996).

Although many studies have demonstrated the ability of ACTH and Ang II to stimulate catecholamine secretion in teleost fish, it has only recently been shown that these secretagogues may represent a functional secondary catecholamine release mechanism *in vivo*. Bernier and Perry (1999) have demonstrated in the rainbow trout that Ang II leads to reproducible and dose dependant increases in DAP, systemic resistance, cardiac output and stroke volume. This is correlated with dose dependant increases in plasma adrenaline but not noradrenaline (Bernier and Perry, 1999; Reid and Perry, 2003). Ang II also stimulated significant catecholamine release both *in vivo* and *in situ* in hypoxic trout experiencing nicotinic receptor desensitisation (Lapner et al., 2000; Lapner and Perry, 2001). Further to this, inhibition of the rennin-angiotensin system (RAS) using the angiotensin converting enzyme inhibitor lisinopril, in desensitised trout, prevented Ang II stimulated catecholamine release during acute hypoxia (Lapner and Perry, 2001) or hypotension (Bernier et al., 1999). Muscarinic or serotonergic receptor blockade had no effect on the ability of desensitised trout to release plasma catecholamines during hypoxia (Lapner and Perry, 2001).

The present study provides some indication that Chinook salmon, too, may rely on non-cholinergic catecholamine secretion pathways during cholinergic receptor blockade. In a preliminary experiment using the PCV preparations previously described, the ability of the chromaffin tissue to release catecholamines in the presence of hexamethonium (a specific nicotinic receptor antagonist), and hexamethonium + atropine (a specific muscarinic receptor antagonist) was investigated. Preparations were perfused with $1x10^{-3}$ mol L^{-1} hexamethonium during which they received a 'one-shot' electrical stimulus for 2 seconds (settings as given in the methods and materials section). Perfusate samples were collected for the 5 minutes following stimulation. The preparation was then perfused with a mixture of $1x10^{-3}$ mol L^{-1} atropine and $1x10^{-3}$ mol L^{-1} hexamethonium and stimulated again. Perfusate samples were collected over the following 5 minutes.

Maximal secretion rates of adrenaline and noradrenaline during hexamethonium perfusion were 44.4pMol min^{-1} and 9.3pMol min^{-1} respectively, indicating that neuronally stimulated catecholamine secretion was not prevented. This suggests a possible role for

non-nicotinic (i.e muscarinic) mediated catecholamine release from chromaffin cells in salmon.

Following neuronal stimulation, secretion of both adrenaline and noradrenaline was significantly increased from basal levels during perfusion with the hexamethonium + atropine combination. Maximal concentrations of adrenaline and noradrenaline were 9.94 $nmol.min^{-1}$ and 7.2 $nmol.min^{-1}$ respectively. Although this represents a single experiment in one fish, Montpetit and Perry (1999) found that while catecholamine secretion during cholinergic receptor blockade was reduced, it was not prevented. This finding reinforces the idea that non-cholinergic mechanisms can stimulate catecholamine secretion in teleost chromaffin cells during cholinergic desensitisation. The most likely candidate for this role is Ang II, which in trout elicits catecholamine release specifically *via* Ang II receptors expressed on the chromaffin cells (Lapner et al., 2000). Even though the concentrations of antagonist used in this experiment and those of Montpetit and Perry (1999) were high, there is no evidence to suggest that either drug at these concentrations causes non-specific side effects on catecholamine secretion (Montpetit and Perry, 1999). Nor do they appear to modify Ang II stimulated release in trout (Bernier and Perry, 1997, cited in Montpetit and Perry, 1999). In fact, it is possible that desensitisation of nicotinic receptors may enhance the chromaffin cells response to non-nicotinic agonists such as Ang II (Lapner et al., 2000).

Given that anaesthetics act to block neuronal sodium channels, and catecholamine release from the chromaffin cells in both fish and mammals is a Ca^{2+} and Na^{+} dependant process (Nagayama et al., 1999; Lapner et al., 2000; Lapner and Perry, 2001), a series of experiments was developed to determine the direct effects, if any, of AQUI-S on neuronally induced catecholamine secretion from salmon chromaffin cells. Results from this series indicate there is no significant effect, either inhibitory or stimulatory, of AQUI-S anaesthesia on neuronally evoked catecholamine secretion from salmon chromaffin cells, *in situ*.

The present study appears to be one of the first to investigate any direct effects of anaesthetics on piscine chromaffin cells *in situ*. Most work on the subject has been carried out on cultured mammalian chromaffin cells. To this end, it has been shown in bovine chromaffin cells, that procaine inhibits cholinergic mediated catecholamine secretion *via* L-type Ca^{2+} channels (Charlesworth et al., 1992; Charlesworth and Richards, 1995). This is due primarily to a decrease in mean channel open time leading to a reduction in agonist evoked inward current. It has also been shown that there is a preferential and direct effect

of anaesthetics on the activation of nicotinic acetylcholine receptors on bovine chromaffin cells (Charlesworth and Richards, 1995). Given these findings, it is not unreasonable to postulate that anaesthetics may have direct effects on catecholamine secretion from piscine chromaffin cells. The fact that in this study AQUI-S appears to have no effect could be due to several possible reasons:

1/ AQUI-S may simply have no effect on the chromaffin cells of salmon. However, it is interesting to note that the 'unidentified' substance seen in HPLC chromatograms of perfusate samples taken during Experiment 3.1b, was associated with perfusion of the chromaffin tissue with AQUI-S and disappeared during perfusion with anaesthetic free saline. Therefore, the anaesthetic must be having at least some direct interaction with either the chromaffin cells or the surrounding tissue. Although this peak remains unidentified, it is likely to be a substance involved either in catecholamine synthesis or metabolism. The compound is not L-DOPA, dopamine or serotonin, nor does its relative mobility correspond to 3,4-dihydroxyphenylglycolaldehyde, which is the metabolite resulting form the activity of monoamine oxidase when noradrenaline is the substrate (M.E. Forster, personal communication).

2/ Although catecholamine release in vertebrates is a Ca^{2+} dependant process, the specific types of voltage dependant Ca^{2+} channels and receptors expressed on the chromaffin cells and involved in the secretion process vary greatly from species to species. For example, cat chromaffin cells express both L- and N-type Ca^{2+} channels, which carry the Ca^{2+} current equally, but the L-type channels dominate exocytosis (Lopez et al., 1994, cited in Nagayama et al., 1999). Bovine chromaffin cells possess L-, N-, P- and Q-type Ca^{2+} channels (Charlesworth et al., 1992; Nagayama et al., 1999), although the L- and Q-type channels dominate exocytosis (Nagayama et al., 1999). In contrast, although the same types of Ca^{2+} channels are expressed on rat chromaffin cells as in bovine cells, it is the L-and N-type that are recruited during exocytosis (Kim et al., 1995, cited in Nagayama et al., 1999).

Although procaine (Charlesworth et al., 1992) and isoeugenodilol, a derivative of isoeugenol (Yeh et al., 2000) have been shown to inhibit Ca^{2+} movements in bovine and rat chromaffin cells, the situation in fish may be different. It is possible that the specific voltage dependant Ca^{2+} channels of piscine chromaffin cells do not share extensive homology with those of mammalian chromaffin cells.

It is also possible that experiments using a different species of fish may yield conflicting results to those presented here. Given the highly variable response of both

mammalian and piscine chromaffin cells to hypoxia (Perry et al., 2000; Lapner and Perry, 2001) it is likely that there may be species specific differences in response to anaesthesia.

3/ The lack of a response to anaesthesia may also represent a limitation of the experimental preparation or an inability of the preparation to release catecholamines.

A similar situation to that with hypoxia may exist with anaesthetic treatment, in that an intact, higher control centre is required before any direct effects of anaesthesia on catecholamine secretion are recognized. There is also a possibility that due to starvation prior to experimentation, preparations derived from unfed animals lacked sufficient catecholamine stores and therefore could not release any upon neuronal stimulation. However, prolonged captivity (2 months) has only small effects on catecholamine storage in rainbow trout and no effect on the ability of chromaffin cells to release catecholamines *in situ* (Reid et al., 1994).

4/ Desensitisation of nicotinic receptor mediated catecholamine release may also account for the low concentrations of catecholamine secretion during neuronal stimulation. From the time of sacrifice of the fish to the end of each experiment at least 110 minutes passed, with the complete series of stimulations and sample collections (1 through 6) taking 90 minutes. At the time of capture and sacrifice fish would have released high concentrations of both adrenaline and noradrenaline. A post-mortem blood sample taken from one fish used in these experiments yielded an adrenaline concentration of 400.2nML^{-1} and a noradrenaline concentration of 175.5nML^{-1}. Although preparations were perfused with saline for 20 minutes prior to the first stimulation, it is possible that high concentrations of circulating plasma catecholamines may have induced nicotinic receptor desensitisation, a phenomenon well documented in mammalian chromaffin cells (Charlesworth et al., 1992; Charlesworth and Richards, 1995; Nagayama et al., 1999) and recently described in trout chromaffin cells (Lapner et al., 2000; Lapner and Perry, 2001). In these studies, desensitisation following maximal nicotinic agonist stimulation occurred within 2 minutes of agonist application, and lasted for ~ 60 minutes. If this is the case in salmon PCV preparations, it might explain why adrenaline values following stim 6, which would occur > 60 minutes after initial stimulation (stim 1), were significantly higher than stim 2-5 inclusive.

The effect of hypoxia on teleost red blood cells.

In vivo, it was seen that anaesthesia with AQUI-S lead to a significant increase in haematocrit and a decrease in mean cell haemoglobin concentration (Chapter 2, Figure 2.8 a&b). Changes in Hct and MCHC were attributed to anaesthetic induced hypoxia, at a

time when there were no significant increases in circulating plasma catecholamines following anaesthetic induction (Chapter 2, Figure 2.6). The whole blood experiments presented in this chapter were designed to reproduce the *in vivo* effects of hypoxia on red blood cell (RBC) volume during moderate hypoxia due to light anaesthesia

The starting values for pH (7.58) and Hct (37.28%) in Chinook salmon prior to hypoxia were lower and higher respectively, compared to values reported for rainbow trout red blood cells taken *via* caudal puncture, of 7.68 and 22.68% (Sørensen and Weber, 1995). Significant increases in plasma pH (pH_e) stimulated by hypoxia have been observed in hypoxic red blood cells (RBC) of rainbow trout (Wood and Simmons, 1994) and are due to the Haldane effect. This is the process in which the release of molecular oxygen from haemoglobin causes protons to bind to the haemoglobin molecule, which then causes a decrease in the intracellular pH of the red blood cell (pH_i). This in turn influences plasma pH. Teleost fish haemoglobins tend to have large Haldane effects and low buffering capacities (Brauner et al., 1996; Jensen et al., 1998). The release of protons upon oxygenation and subsequent increase in pH_i is a vital step in CO_2 excretion by the teleost RBC. An increase in plasma P_{CO_2} accompanied by a fall in plasma pH (pH_e) will cause a reduction in the haemoglobin-oxygen binding affinity *via* the Root effect (Nikinmaa, 1997). The Bohr effect also enhances O_2 offloading at the tissues due to an increased P_{CO_2} within the capillaries. The majority of Bohr protons are released between 60-100% Hb-O_2 saturation, indicating the ability of fish blood to fully exploit the Haldane effect and enhance CO_2 excretion (Brauner et al., 1996).

In teleost fish blood there is a large pH gradient across the red blood cell, with H^+, Cl^- and HCO_3^- passively distributed across the membrane according to a Donnan equilibrium (Jensen, 1986; Malapert et al., 1997; Gilmour, 1998). The mechanism through which protons are passively equilibrated across the red blood cell is called the Jacobs-Stewart cycle (Figure 3.7). Disturbance of the acid-base balance causes a redistribution of anions by the band 3 anionic exchanger, accounting for the linear relationship seen under physiological conditions between pH_i and pH_e (Fiévet and Motais, 1991; Malapert et al., 1997). Metabolites produced within the body tissues create a concentration gradient for metabolites, specifically CO_2, across the RBC membrane. CO_2 is transported in the blood mainly as bicarbonate (HCO_3^-) with 98-99% of the CO_2 entering the gill vasculature in this form (Heming, 1984, cited in Brauner and Randall, 1996; Gilmour, 1998, Maffia et al., 2001). However, erythrocytes themselves contain only approximately 15% of the total

CO_2 content of the blood (Nikinmaa and Vihersaari, 1993). Following diffusion into the RBC from the tissues, CO_2 is hydrated to HCO_3^- according to the following equation:

$$H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

This process is catalysed by the zinc metalloenzyme carbonic anhydrase (CA), which is present in high concentrations within the red blood cell and also in the gills (Maffia et al., 2001). The amount of HCO_3^- formed at a given P_{CO_2} increases with an increase in pH_i (Nikinmaa, 1997). Binding of Bohr protons to deoxygenated Hb favours the formation of HCO_3^- as per the above equation, which is then removed from the RBC in exchange for Cl^- ions, *via* the band 3 anion exchanger present in the RBC membrane (Nikinmaa, 1997; Gilmour, 1998; Nikinmaa and Salama, 1998; Walsh et al., 1998). This is also known as the 'chloride shift' (Gilmour, 1998). To maintain electrochemical neutrality, Na^+ ions must move into the cell to balance the increasing Cl^- concentration. This net entry of Na^+ and Cl^- induces the entry of osmotically obliged water into the RBC causing the cell to swell (Fiévet and Motais, 1991; Nikinmaa, 1997; Gilmour, 1998; Nikinmaa and Salama, 1998; Walsh et al., 1998).

This process would explain the rise in pH_e seen in the present study upon exposure of RBC to hypoxic conditions *in vitro*. In red blood cells from rainbow trout, hypoxia elevates CO_2 excretion by ~20% compared to normoxic conditions (Wood and Simmons, 1994) and increases in pH_e at a constant P_{CO_2} (as in the present study) are associated with an elevation in plasma HCO_3^- (Wood and Simmons, 1994). It is likely, that in the present study, if measured, plasma CO_2 content would also have been elevated as it was in rainbow trout blood (Wood and Simmons, 1994) given that a tonometer is an open system.

The concomitant increase in Hct and decrease in MCHC seen during hypoxia *in vitro* and changes in Hct and MCHC seen *in vivo* during light anaesthesia, can be attributed to erythrocyte swelling. This increase in cell volume effectively dilutes the haemoglobin molecule (decreasing MCHC) and increases Hct. Indeed, the mean cell volume of hypoxic trout red blood cells is significantly greater than the mean cell volume of oxygenated trout RBC (Sørensen and Weber, 1995). The functional advantage of erythrocyte swelling is the dilution of organic phosphates that can interfere with the binding of Bohr protons to the haemoglobin molecule. Organic phosphates such as ATP or GTP modulate haemoglobin function in fish RBC by 2 interactions; 1/ by directly and preferentially binding to deoxygenated haemoglobin; 2/ by having an indirect effect on pH_i (Nikinmaa and Salama, 1998). Allosteric interactions between NTPs and haemoglobin

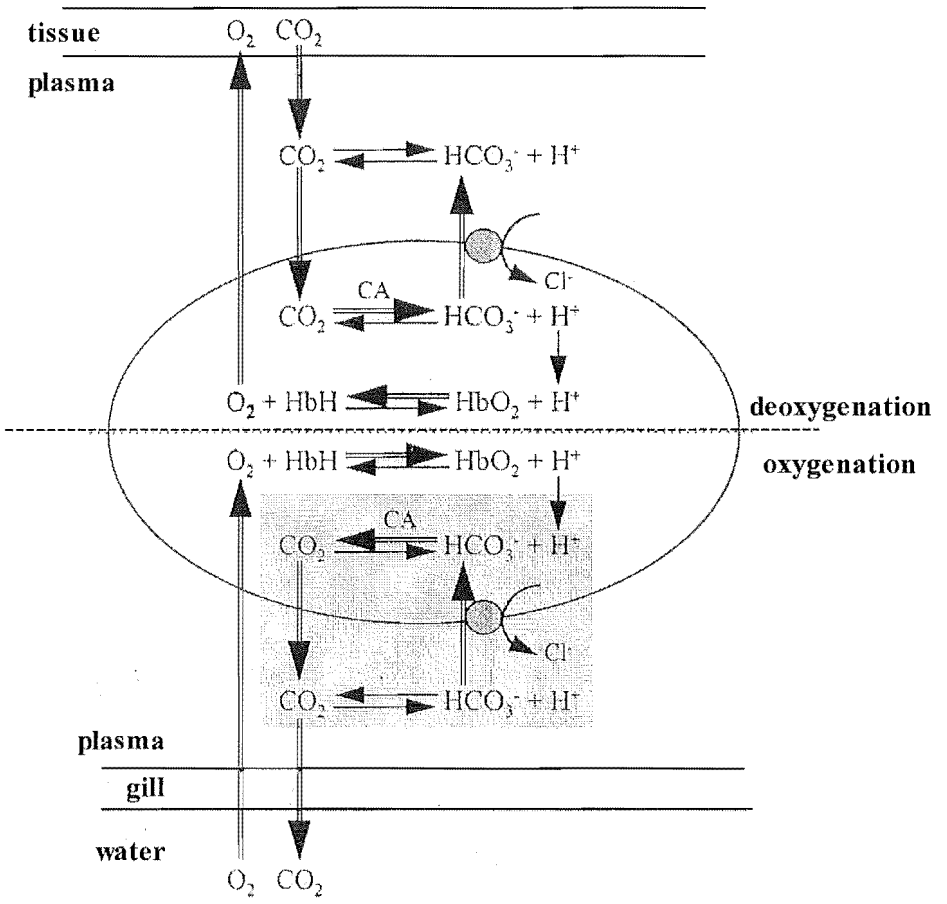


Figure 3.7 Schematic representation of the carbon dioxide (CO_2) excretion pathway in most teleost fish red blood cells (rbc). As Hb is deoxygenated at the tissues, CO_2 diffuses into the red blood cell were it is hydrated to HCO_3^- and H^+, a process catalysed by carbonic anhydrase. HCO_3^- leaves the cell in exchange for Cl^- via the band 3 anion exchanger. At the gill, CO_2 diffuses into the surrounding water driving the dehydration of HCO_3^- within the rbc. HCO_3^- entry into the rbc is enhanced. Hb=haemoglobin, O_2=oxygen, CA=carbonic anhydrase HCO_3^- =bicarbonate. The grey box indicates the Jacobs Stewart cycle. See text for further details. (Taken from Gilmour, 1998).

lead to a reduction in haemoglobin-oxygen binding affinity (Nikinmaa and Salama, 1998; Gilmour, 1998)

The predominant organic phosphate present in fish erythrocytes varies greatly from species to species, but is always either ATP or GTP. In salmonid fish, ATP is the predominant organic phosphate affecting haemoglobin function, binding at the entrance to the central cavity between the two β polypeptide chains of haemoglobin in the deoxygenated (T) state. Binding of ATP stabilises the T state by the introduction of additional bonds (Jensen et al., 1998) e.g ATP readily complexes with magnesium ions

(Bunn et al., 1971, cited in Nikinmaa and Salama, 1998). The interaction of ATP and haemoglobin reduces the Hb-O_2 binding affinity for a given PO_2. Erythrocyte swelling effectively dilutes both organic phosphates and haemoglobin, thereby reducing allosteric reactions between them and enhancing Hb-O_2 binding affinity (Gilmour, 1998; Nikinmaa and Salama, 1998; Jensen, 1998).

Under conditions of extreme stress such as environmental hypoxia or metabolic acidosis due to severe exercise, optimal Hb-O_2 affinity is mediated by the release of plasma catecholamines into the circulation. Stimulation of the β-adrenergic mediated Na^+/H^+ exchanger (βNHE) on the teleost RBC membrane protects the erythrocyte against significant decreases in pH_i that may occur during severe stress (Malapert et al., 1997; Nikinmaa, 1997). The efflux of protons into the plasma increases pH_i, thereby increasing oxygen uploading at the gill (due to the Root effect) in the face of acidosis.

Na^+/H^+ exchangers are integral plasma membrane proteins that have been identified in the plasma membrane of nearly all eukaryotic cells (Bianchini and Pouyssegur, 1994). Five isoforms have been characterised to date (NHE-1–NHE-5) and their activity and level of expression can be modulated greatly by a variety of stimuli such as growth factors, tumour promoters, hormones and chronic cellular acidification (Bianchini and Pouysségur, 1994). They can also be stimulated by changes in cell volume. The βNHE of teleost red blood cells shares significant molecular homology with NHE-1, 74% for the transmembrane domain and 48% for the cytoplasmic domain (Borgese et al., 1992, cited in Bianchini and Pouysségur, 1994). The salmonid βNHE however, is different from other known isoforms in several important aspects that may reflect the specificity of its function. Firstly, the teleost βNHE is the only isoform known to be activated via cAMP. Secondly, unlike other isoforms, it is not activated *via* extracellular acidification alone. Thirdly, its activation is rapidly followed by its desensitisation (Guizouarn et al., 1993; Bianchini and Pouysségur, 1994, Gilmour et al., 1994, Malapert et al., 1997; Gilmour, 1998).

Unlike other Na^+/H^+ exchangers in vertebrates, which are primarily concerned with cell volume regulation during osmotic shock, the trout Na^+/H^+ exchanger can only be activated under normal physiological conditions by catecholamine hormones (Fievet and Motais, 1991; Malapert et al., 1997). This hormonal stimulation is so great, that the maximum Na^+ influx of adrenergically stimulated trout βNHE is 10-100 times greater than in other vertebrate red blood cells (Fievet and Motais, 1991, Guizouarn et al., 1993).

Binding of catecholamines to adenylate-cyclase coupled β-adrenoceptors on the surface of the RBC stimulates formation of the second messenger cAMP (Figure 3.8). In turn, cAMP initiates a phosphorylation pathway resulting in the activation of the cAMP-dependant Na^+/H^+ exchanger (Guizouarn et al., 1993; Bianchini and Pouysségur, 1994; Gilmour et al., 1994, Gilmour, 1998). Due to the greater potency of noradrenaline on proton extrusion from the trout RBC (Tetens and Lykkeboe, 1988; Reid and Perry, 1991), it has been generally accepted that the β-adrenoceptor subtype involved in stimulation of the exchanger is of the β_1 (noradrenaline) subtype. However, recent cloning of β-adrenoceptors from rainbow trout have indicated that the βNHE may be specifically controlled by a tentatively named 'β_{3b}'-adrenoceptor, which is homologous to the well characterised mammalian β_3 adrenoceptor and is highly expressed in the trout red blood cell (Nickerson et al., 2003). Identification of a single category of β-adrenoceptor binding sites on the trout RBC membrane, coupled with expression of the 'β_{3b}'-adrenoceptor, provides significant evidence for the control of trout RBC βNHE activity *via* signalling through this new receptor subtype and not through a β_1-subtype as has been previously proposed (Nickerson et al., 2003).

Activation of the trout βNHE via catecholamines causes a net efflux of protons out of the erythrocyte, coupled with a net influx of Na^+ (Figure 3.8). This increases the intracellular pH and decreases the extracellular pH. The decreasing pH_e favours the dehydration of plasma HCO_3^- to CO_2, although due to the absence CA in the plasma, this reaction is slow (Gilmour, 1998). The CO_2 produced can diffuse across the cell membrane into the RBC where it is converted to protons and HCO_3^- via CA (Gilmour, 1998; Nikinmaa and Salama, 1998). The Na^+/H^+ exchanger capacity for proton excretion far exceeds CO_2 diffusion and hydration in the RBC (Gilmour, 1998). Again, HCO_3^- is exchanged for Cl^- across the band 3 anion exchanger, Na^+ entry is followed by osmotically obliged water and the cell swells (Fiévet and Motais, 1991; Nikinmaa, 1997; Gilmour 1998; Nikinmaa and Salama, 1998; Walsh et al., 1998). Na^+ entry also stimulates the transmembrane K^+/Na^+ pump which in turn increases the overall energetic demands of the rbc, causing a decrease in intracellular NTP concentration (e.g ATP and/or GTP) and, further enhancing Hb-O_2 binding. Thus activation of the βNHE effectively 'uncouples' pH_i and pH_e. Although reliant on catecholamine hormones for transition to a functional state, prolonged exposure of the βNHE to high levels of circulating catecholamines results in a desensitisation of the signalling cascade initiating βNHE function. This may in part be

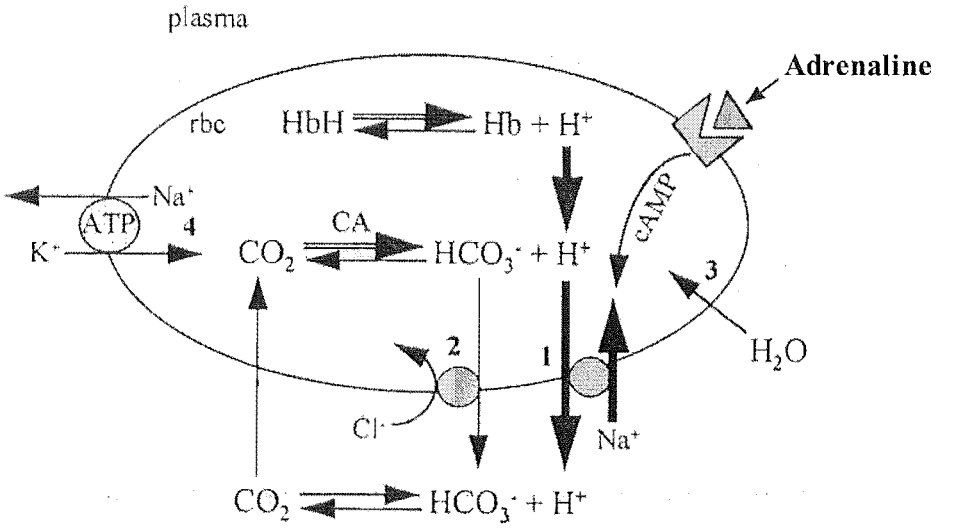


Figure 3.8 Adrenergic stimulation of cell swelling in a teleost red blood cell (rbc). Adrenaline (or noradrenaline) binds to a β-adrenoreceptor on the cell membrane stimulating cAMP signalling cascade and activation of the Na^+/H^+ antiporter (βNHE). Protons released from Hb or created by CO_2 hydration are pumped out of the rbc (**1**) The increasing [HCO_3^-] stimulates Cl^-/HCO_3^- exchange (**2**). Osmotic water enters the rbc, causing the cell to swell (**3**) while the increasing internal [Na^+] activates Na^+-K^+ exchange (**4**). This increases energy consumption leading to a decrease in intracellular [NTP]. See text for further details. (Modified from Gilmour, 1998).

due to a down regulation of the red blood cell β-adrenoceptors (Gilmour et al., 1994; Malapert et al., 1997).

Given the high concentrations of catecholamines present in both Chinook salmon and Snapper plasma following deep anaesthesia, it is likely that changes in Hct and MCHC (Chapter 2, Figure 2.8 and 2.13, respectively) seen involved the activation of the βNHE. This is reinforced in salmon given the positive regression between plasma catecholamines and Hct (Figure 2.11a&b).

Hypoxia has been shown to significantly sensitise the teleost βNHE to any given concentration of catecholamine, possibly partially due to an increase in the number of cell surface β-adrenoceptors (Reid and Perry, 1991) and an increase in the sensitivity of the signalling pathway to cAMP (Reid et al., 1993). This oxygen effect appears not to be correlated with RBC metabolism, but with the quaternary structure of haemoglobin (Motais et al., 1987, cited in Fiévet and Motais 1991), given that βNHE activity is enhanced at pH values stabilising the deoxy 'T' state of haemoglobin (Salama and Nikinmaa, 1989). There is also evidence that some other pH sensitive haemoprotein other

than haemoglobin may play an essential role in the cellular O_2 sensing mechanism in trout red blood cells (Berenbrink et al., 2000).

Prolonged stress with concurrent elevation of plasma cortisol levels also has an effect on the βNHE. EC_{50} values for noradrenaline stimulation of the exchanger were significantly lower in trout blood from fish chased to exhaustion for 7 days (Perry et al., 1996). *In vitro* experiments showed that during hypoxia, although the number of cell surface β-adrenoceptors was actually 30% lower in blood from stressed fish with high cortisol levels, binding affinity for catecholamines was significantly enhanced compared to normoxia (Reid and Perry, 1991; Perry et al., 1996). It is suggested that this increase in sensitivity to catecholamines may be due to pre-adaptation of the RBC by cortisol to other physiological signals, allowing low-affinity internalised β-adrenoceptors to be converted to high affinity receptors and 'recycled' back to the cell surface (Reid and Perry, 1991).

In vivo it was seen that anaesthesia induced erythrocyte swelling via both adrenergic (Series 2.3) and non-adrenergic (Series 2.1) mechanisms. These non-adrenergic mechanisms were replicated *in vitro,* showing hypoxia was the proximate stimulus for erythrocyte swelling in these experiments. The ability of Chinook salmon red blood cells to respond to both hypoxic and adrenergic stimulation was maintained during anaesthesia, therefore indicating a lack of any apparent effect of even high concentrations of anaesthesia in the normal functioning of the RBC.

AQUI-S appeared not to have a direct effect on catecholamine secretion in the perfused posterior cardinal vein of Chinook salmon. However, it is unknown whether there may be a direct effect on catecholamine release stimulated by the O_2 chemoreceptors postulated by Reid and Perry (2003). AQUI-S is known to be an irritant to fish (personal observation and Hill, 1999) and at high concentrations it is possible it stimulates nociceptors on the gills of salmon (Hill, 1999). If this is the case, the possibility exists that AQUI-S at high concentrations may also affect the O_2 chemoreceptors on the fish gill. However, elucidation of the exact mechanism of signal transduction from chemoreceptors to the chromaffin tissue is required before modulation by external stimuli can be investigated effectively.

Chapter 4

In vitro and *in situ* effects of anaesthetics on the blood vessels of Chinook Salmon.

Introduction

Vascular tone in vertebrates is a compromise between vasoconstrictor and vasodilator pathways. Vasomotor responses to neural, metabolic and physical factors vary not only between different vessels in different vascular beds, but also along the same vascular bed (Hill et al., 2001). In the absence of external neural metabolic or environmental stimuli, blood vessels also display intrinsic activity in response to changes in transmural pressure. An increase in transmural pressure causes constriction whereas a decrease in pressure results in dilation (Davis and Hill, 1999). This is known as the myogenic response and is inherent to smooth muscle cells. The myogenic response is crucial in the development of basal vascular tone and autoregulation of blood flow and capillary hydrostatic pressure (Davis and Hill, 1999). Blood vessels also respond to external stimuli such as neurotransmitters, circulating vasoactive hormones and environmental stimuli. For example, low pH and O_2 tension and high CO_2 tension tends to relax vessels, whereas the reverse conditions tend to cause constriction (Baum, 1977; Hill et al., 2001; Smith et al., 2001).

The relative resistance of each vascular bed will determine the proportion of cardiac output it receives and the state of the vascular smooth muscle contraction constitutes the major determinant of resistance (Baum, 1977; Satchell, 1991; Hill et al., 2001). In fish as in mammals, arteries and arterioles are presumed to be the major source of resistance (Olson, 1998b) although it is the venous system that displays the greatest capacitance (Conklin and Olson, 1994).

In mammals, most blood vessels are richly innervated with sympathetic nerves and stimulation or administration of noradrenaline results in vasoconstriction of vessels (Baum, 1977; Hill et al., 2001). However, sympathetic stimulation may affect vessels in

different organs or even within an organ, differentially (Baum, 1977). The situation in teleost fish is not dissimilar. Adrenergic nerves innervate systemic blood vessels of most fishes (Satchell, 1991). During severe hypoxia blood vessels also react to humoral catecholamines secreted from the chromaffin tissue (Wendelaar Bonga, 1997; Perry and Bernier, 1999).

In mammals, general anaesthetics are well known to cause a drop in central blood pressure and hypoxia via depression of cardiovascular control centres in the brain, and possible direct actions on blood vessels (Angel, 1980; Urban, 2002). In fish, studies have concentrated on changes in blood pressure *in vivo* (refer Chapter 2 for references) with *in vitro* and *in situ* studies primarily investigating anaesthetic effects on the heart (Randall, 1962; Pierce and Pierce, 1967; Ryan et al., 1993) kidney (Soivio et al., 1974) or gill (Fromm et al., 1971; Soivio and Hughes, 1978; Hill, 1999). No study seems to have specifically addressed the impact of anaesthesia on muscle perfusion. Hill (1999), studied afferent and efferent arteries from both *Oncorhynchus tshawytscha* and spotty, *Notolabrus celidotus* and found both MS222 and AQUI-S were potent vasodilators of afferent and efferent branchial arteries. If one of the main actions of catecholamines during hypoxia is to increase central blood pressure and anaesthetics antagonise this action via direct effects on the blood vessels themselves, there may be serious consequences to the fish with regard to perfusion patterns of the musculature and the gill.

Methods and Materials

Series 4.1 Effects of anaesthetics on the vascular response of Chinook salmon to adrenaline *in vitro*.

Experiment 4.1a Effects of AQUI-S and MS222 on the response of the hepatic portal vein to adrenaline *in vitro*.

Chinook salmon (*Oncorhynchus tshawytscha*, mean weight 794.4 ± 282.4g (±1SEM) range 1124.2–351.9g, n=14) were transported from Isaacs Salmon farm (McLeans Island, Christchurch, New Zealand) to the University of Canterbury and held in a large darkened fibreglass tank (1.5 x 1.5 x 1.5m) supplied with constantly flowing fresh artesian water. Water temperature ranged from 12-13°C throughout the experimental period and fish were maintained on a 12:12 hour light:dark regime.

When required, fish were removed from the holding tank and killed with a sharp blow to the head. 1ml of heparinized freshwater teleost saline was injected into the dorsal aorta via the roof of the mouth and allowed to circulate for one minute. Fish were then placed ventral side up on an operating sling and an incision made running from the pectoral to pelvic fins. The oesophagus, stomach and most of the gut were removed to enable easy visualisation of the hepatic portal vein. This vessel was chosen due to its relatively large size and easy identification in situ. The vein and its surrounding tissue was then removed and placed in a petri dish containing fresh water teleost saline. Under magnification the tissue surrounding the vein was then carefully removed via blunt dissection and the vessel cut into pieces approximately 4mm long.

A schematic diagram of the myography set-up is shown in Figure 4.1. Vessel rings were threaded onto two small wire 'clips'. These 'clips' were a triangle shape with the wire forming the flat 'base' being threaded through the length of the lumen of the vessel ring (Figure 4.1. (a)) (Olson et al., 2001). This allowed a measure of tension change along the length of the vessel ring, rather than at only one specific point. The 'peak' of the triangle was further modified to form a loop that could be anchored in the myography chamber. The vessel was then placed in a myography chamber. Changes in vessel tension were measured using UGO Basile (7003) isometric force transducers, connected to a Gould transducer amplifier (Model 13-4615-50) electronically linked to a chart recorder (Yokogawa LR 4110 E myograph with digital acquisition, Japan, Figure 4.1.(b)). The vessel was held fixed on one side by a hook in the bottom of the myograph well, while the opposite site was attached to the arm of the myograph transducer, via a thread tied to the loop of the wire 'clip', threaded through the vessel lumen. The well was filled with 20mL of fresh water teleost saline (see Chapter 2) and constantly but gently (so as not to knock the vein) gassed with air. The wells were water jacketed and held at a constant temperature of 11°C. The transducer arm was then slowly and carefully moved up away from the vessel until there was tension on the thread. The vessels were then left until tension became stable. This was usually about 20 minutes. The myograph transducer was calibrated to either 250mg or 500mg range, depending on the starting tension of the vessel.

Following the equilibration period, an aliquot of adrenaline was added to the saline in the myography well to give a final concentration of $1x10^{-7}$M. Once the maximal response to the adrenaline treatment had been observed (usually 5-10 minutes) the adrenaline was washed out thoroughly (3 cycles of washing and flushing with fresh saline were performed) and the vessel tension left to stabilize for a further 20 minutes. After this

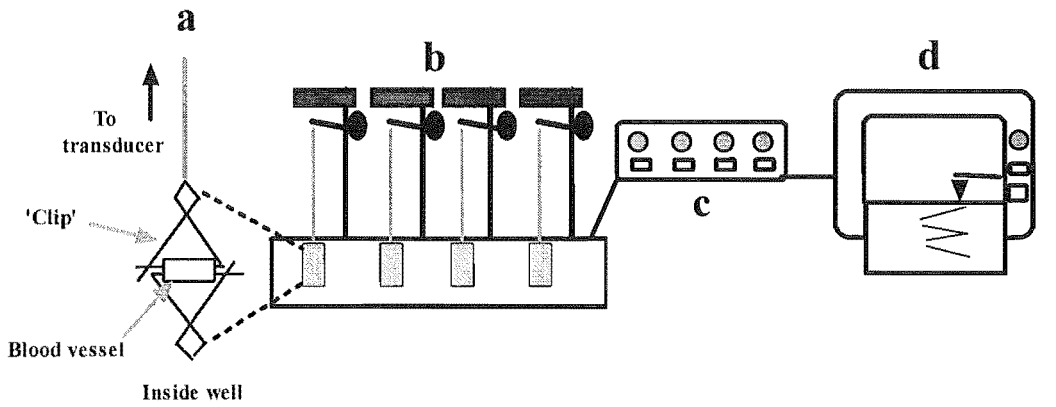


Figure 4.1. Schematic diagram of the myography set-up used in Experiment 4.1.a and Experiment 4.1.b. Vessel rings were threaded onto two 'clips' along the length of the lumen (a) and placed in myograph wells filled with saline. Tension was measured with isometric force transducers (b) connected to a transducer amplifier (c). This in turn was connected to a Yokogawa myograph (d), which produced both a digital and analogue record of changes in vessel tension. See text for further details.

time, an aliquot of anaesthetic was added to the well to give a final concentration of either 60ppm AQUI-S (n=10) or 100ppm MS222 (n=11). After 5 minutes, a further aliquot of adrenaline (to give $1x10^{-7}$M) was added to the vessel, in the presence of the anaesthetic. Again, following the maximal response to adrenaline the saline was washed out as above, and the vessel tension left to stabilize. Finally, the vessel was treated with a third aliquot of adrenaline, in the absence of anaesthetic.

Controls were treated with the same protocols as described above however, they were not exposed to anaesthetic prior to the second adrenaline treatment, or at any point throughout the experiment.

Data was analysed using repeated measures ANOVA with Bonferroni post-hoc test, to compare vessel responses to adrenaline prior, during and following anaesthetic exposure both within a treatment group and between groups.

Paired Students 't' test was used to determine whether anaesthetic exposure alone had a significant effect on the vessel.

Unless otherwise stated, significance levels were set at $P \leq 0.05$. All statistical analyses were performed using GraphPad Prism, version 3.01 for Windows, San Diego, California, USA.

Experiment 4.1.b. Effects of AQUI-S and MS222 on the response of the hepatic portal vein to adrenaline *in vitro*; construction of adrenaline concentration curves.

Hepatic portal vein 'rings' were obtained from fish also used in Experiment 4.1.a. and prepared as described above. Following the initial stabilisation period anaesthetic was added to the well to give a final concentration of either 60ppm AQUI-S (n=9) or 100ppm MS222 (n=10). After 5 minutes an aliquot of adrenaline was added to give a final concentration of $1x10^{-9}$M adrenaline. Once the maximal response to this concentration was observed (usually within 5 minutes) a further aliquot of adrenaline was added to give a final concentration in the well of $1x10^{-8}$M adrenaline. Again, when the maximal response was observed, an aliquot of adrenaline was added to give a final concentration of $1x10^{-7}$M. Proceeding in this way, a concentration curve for adrenaline was constructed ranging from $1x10^{-9}$M to $1x10^{-3}$M in the presence of either AQUI-S or MS222 anaesthetic.

Controls were treated with the same protocols as described above. However, they were not exposed to anaesthetic at any point throughout the experiment.

Sigmoidal dose response curves were constructed using GraphPad Prism and $LogEC_{50}$ values determined. These values were then analysed using repeated measures ANOVA with Bonferroni post-hoc test to compare differences between vessels treated with each anaesthetic and control vessels. Unless otherwise stated significance levels were set at $P \leq 0.05$. All statistical analyses were performed using GraphPad Prism, version 3.01 for Windows, San Diego, California, USA.

Series 4.2. Vasoactive effects of anaesthetics on the vasculature of Chinook salmon tail, *in situ.*

Holding conditions for fish used in Series 4.2. varied. For some experiments, Chinook salmon (*Oncorhynchus tshawytscha*) were transported from Isaacs Salmon farm (McLeans Island, Christchurch, New Zealand) to the University of Canterbury and held in a large darkened fibreglass tank (1.5 x 1.5 x 1.5m) supplied with constantly flowing fresh artesian water. Water temperature ranged from 12-13°C throughout the experimental period and fish were maintained on a 12:12 hour light:dark regime. Fish were not fed for 2

weeks leading up to or at any stage during the experimentation period. Experiments were carried out at the University of Canterbury.

However, due to changes in the aquarium facilities at the University of Canterbury, and concerns about the water quality, some experiments were carried out at the salmon hatchery at Isaacs Salmon Farm, (McLeans Island, Christchurch, New Zealand). In this case, Chinook salmon (*Oncorhynchus tshawytscha*) donated by Isaacs Salmon Farm were transferred to their on site hatchery in chilled, oxygenated water. Fish were held in a large round indoor tank (1.5m diameter x 1.5m depth), with constantly flowing fresh artesian water. Both the tank water and that used in experiments came from the same source and had a constant temperature of 12°C. Fish were not fed for 2 weeks leading up to or at any stage during the experimentation period. Experiments were carried out on site at the Salmon Hatchery.

Saline used in this experimental series consisted of Hepes buffered fresh water teleost saline[4] containing 4% PVP (MW = 60 000) and 0.2% Bovine Serum Albumin (Fraction V low endotoxin BSA, GibcoBRL, Life Technologies Ltd., Auckland, New Zealand). Saline was filtered through a double layer of filter paper (G.E. No.1, 9.0cm total diameter) before use. The high concentration and high molecular weight of PVP made it impractical to filter through a Millipore system. Adrenaline was added immediately prior to use to give a final concentration of $1x10^{-7}$M in each 20mL saline reservoir.

Experiment 4.2.a. Vasoactive effects of AQUI-S on the vasculature of the perfused tail of Chinook salmon.

A schematic diagram of the tail perfusion set up is shown in Figure 4.2. When required, Chinook salmon (*Oncorhynchus tshawytscha*, mean weight 1001.7 ± 398.6g, (± 1 SEM), range 551.2-1846.1, n=12) were removed from the holding tank and killed with a sharp blow to the head. 1ml of heparinized freshwater teleost saline was injected into the dorsal aorta via the roof of the mouth and allowed to circulate for approximately one minute. Using a sharp knife, the tail was removed just proximal to the vent and an inflow cannula (1.5mmOD:1mmID, portex tubing), inserted into the dorsal aorta. The caudal vein

[4] Masses for 1 litre solution:
NaCl 7.000g, KCl 0.300g, $MgSO_4.7H_2O$ 0.293g, $CaCl_2.2H_20$ 0.225g, Glucose 1.000g, HEPES 0.715g, NaHEPES 1.820g, PVP 40.000g, BSA 2.000g.

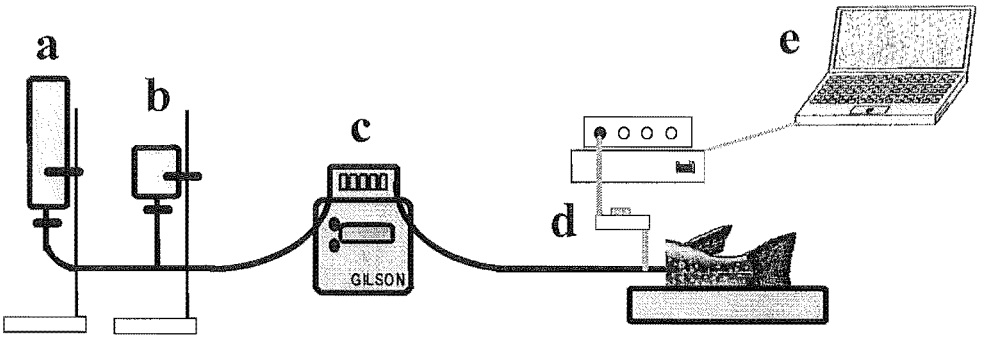


Figure 4.2. Schematic diagram of the tail perfusion set-up used in Experiment 4.2.a and Experiment 4.2.b. Anaesthetic free saline was pumped from a reservoir (**a**) by a peristaltic pump (**c**) into the DA of Chinook salmon tails. A pressure transducer (**d**) connected to a PowerLab amplifier allowed continuous recording of relative pressures within the tail. Pressure readings (cm H_2O) were recorded digitally on a laptop computer (**e**). During perfusion with anaesthetic, flow was changed via a tap system to a small reservoir (**b**) containing the appropriate anaesthetic concentration. See text for further details.

was then cannulated and this acted as an outflow cannula. HEPES buffered fresh water teleost saline containing a physiological concentration of adrenaline ($1x10^{-7}$mM) was pumped into the dorsal aorta from a large reservoir using a constant flow peristaltic pump (Gilson miniplus 3) at a rate of 1ml/min. Saline was already being pumped through the inflow cannula prior to insertion into the dorsal aorta in order to minimise the possibility of passing air bubbles through the preparation.

At approximately 4cm prior to its insertion into the dorsal aorta, the inflow cannula was linked via a 3-way connector to a disposable pressure transducer (PVB 6003, Surgicare Ltd., Victoria, Australia), allowing measurement of relative pressure changes within the tail preparation. The transducer was in turn linked to a digital amplifier (PowerLab 400 with Quad bridge amplifier, ADInstruments, Dunedin, New Zealand) connected to a laptop computer. Data was collected using PowerLab 'Chart', version 3.4, for Windows (ADInstruments, Dunedin, New Zealand).

The preparation was perfused with aerated fresh water teleost saline and left to equilibrate for at least 25 minutes until the pressure became stable. After this time flow was switched via a 2-way tap to a small reservoir (approximately 18ml volume) containing 40ppm AQUI-S in saline. This was pumped into the tail for 15 minutes. At the

end of this time, flow was changed to normal, anaesthetic free saline for a 20-25 minute recovery period. Following this recovery period, flow was changed again to the small reservoir, this time containing 60ppm AQUI-S in saline, for a further 15 minutes. Again after this time, the preparation was perfused with anaesthetic free saline for a 20-25 minute recovery period before the next anaesthetic concentration was tested. This process was repeated for 80ppm AQUI-S and finally 200ppm MS222.

To gauge the potential for vessel reactivity the preparation was perfused with saline containing a final concentration of $1x10^{-5}$M adrenaline at the end of the experimental procedure. This is not a physiological concentration and therefore should elicit an obvious decrease or increase in vessel resistance.

Throughout the experiment, all saline whether anaesthetic free or containing anaesthetic, was maintained at 11°C and contained $1x10^{-7}$M adrenaline to maintain basal venous tone. Changes in relative pressure within the perfused tail preparation were continuously recorded during the experiment.

Experiment 4.2.b. Vasoactive effects of MS222 on the vasculature of the perfused tail of Chinook salmon.

When required, Chinook salmon (*Oncorhynchus tshawytscha*, mean weight 1328.1 ± 546.2g, (± 1 SEM) range 527.1-1754.3g, n=11) were removed from the holding tank and killed with a sharp blow to the head. 1ml of heparinized freshwater teleost saline was injected into the dorsal aorta via the roof of the mouth and allowed to circulate for approximately 1 minute. The tail was removed and perfusion carried out using protocols identical to those described for Experiment 4.2.a. However, in these experiments the effects of increasing concentrations of MS222 were tested in the following order; 50ppm MS222, 100ppm MS222, 200ppm MS222. After these concentrations had been tested, the tail was perfused with 80ppm AQUI-S for a further 15 minutes followed by perfusion with a maximal dose of adrenaline ($1x10^{-5}$M).

All other experimental conditions and saline used were identical to those in Experiment 4.2.a.

Statistical analysis for Series 4.2.

During individual experiments, pressure tended to gradually rise over time, probably due to oedema of the tissues. Determining the slope of the rise by regression analysis and subtracting this from the pressure trace corrected for any gradual increases over time.

Because starting pressure varied greatly between fish, data were expressed as a fraction of the maximum pressure obtained within each experiment. The data was then arcsin transformed using a commercial statistic and graphing package (GraphPad Prism version 3.01 for windows, San Diego, California, USA) prior to any statistical analyses. Unless otherwise stated, $P \leq 0.05$ was taken to indicate a statistically significant difference.

Repeated measures ANOVA with Bonferroni post-hoc test comparing selected groups was used to compare the following:

- Effects of each anaesthetic concentration on pressure within the preparation compared to the control period 10 minutes prior to and 10 minutes following anaesthetic exposure.
- Effects of increasing anaesthetic concentrations versus lower anaesthetic concentrations.
- Effects of final addition of $1x10^{-5}$M adrenaline

Unpaired Student's 't' test was used to compare the following:

- Effects of matched concentration of AQUI-S and MS222 (i.e 40ppm AQUI-S versus 50ppm MS222, 60ppm AQUI-S versus 100ppm MS222 etc.)
- Effects of 200ppm MS222 in fish previously exposed to AQUI-S versus in fish exposed only to MS222, and vice versa for 80ppm AQUI-S.

Analysis of early experiments indicated a significant effect of holding time prior to experimental use of the fish on the response of tail preparations to anaesthetic exposure. To investigate this further, fish were divided into two groups; 'New' fish that were held either at the University of Canterbury or Isaacs Salmon farm hatchery for no longer than 1 week after initial transport and 'Old' fish that were held for more than one week and up to

4 weeks at either location following initial transport. All other experimental procedures were identical to those described above in Experiment 4.2.a and Experiment 4.2.b.

Unpaired Students 't' test was used to compare the effect of both anaesthetics between 'Old' and 'New fish'.

For all statistical analyses, significance levels were set at $P \leq 0.05$, unless otherwise stated.

Results

Series 4.1 Effects of anaesthetics on the vascular response to adrenaline *in vitro*.

Experiment 4.1a. Effects of AQUI-S and MS222 on the response of the hepatic portal vein (HPV) of Chinook salmon to adrenaline *in vitro*.

Data are presented as the change in vessel tension, from resting, in mg per mg wet weight of tissue. The resting tension was taken as the tension at the end of the 20-minute period following each wash and immediately prior to addition of adrenaline or anaesthetic. Graphs showing mean change in tension on exposure to adrenaline for each treatment group and control are shown in Figure 4.3.a-c.

The addition of AQUI-S or MS222 to the vessel resulted in a significant ($P \leq 0.05$, n=10 (AQUI-S), n=11 (MS222)) vasodilation of the HPV when compared to control vessels. This dilation was also significantly different to any change in tension caused by the addition of adrenaline prior to anaesthetic addition ($P \leq 0.05$, n=10 (AQUI-S), n=11 (MS222)). There was no significant difference between the effect of each anaesthetic on vessel tension.

There was no significant difference between the response to adrenaline before, during or after anaesthetic exposure. In other words, exposure to either anaesthetic neither enhanced nor inhibited the ability of the HPV to react to adrenaline, compared to control vessels.

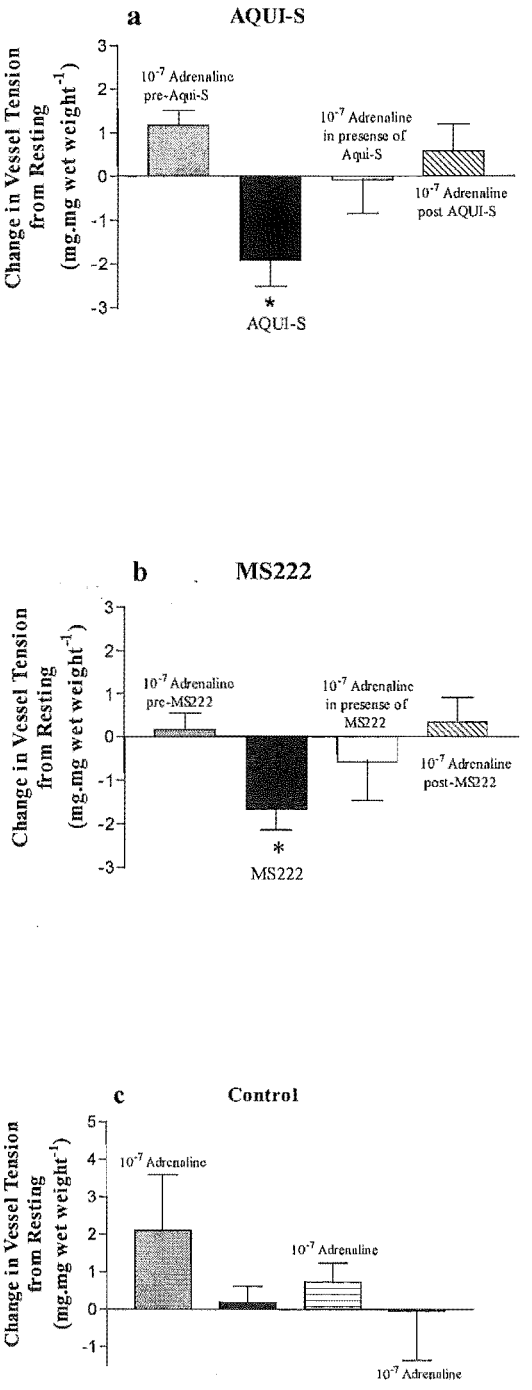


Figure 4.3.a-c. Graphs showing mean change in tension (mg.mg wet weight^{-1}) of the hepatic portal vein (HPV) of the Chinook salmon after treatment with $1x10^{-7}$M adrenaline before during and after exposure to either 60ppm AQUI-S or 100ppm MS222 anaesthesia. * indicates a significant difference ($P \leq 0.05$) from control values.

Experiment 4.1b. Effects of AQUI-S and MS222 on the response of the hepatic portal vein to adrenaline *in vitro*; construction of adrenaline concentration curves.

Fitting of Sigmoidal dose response curves to individual data sets was complicated by the presence of 2 receptor systems for adrenaline (α and β), which have antagonistic effects on vessel tension (contraction and relaxation, respectively). Only vessels that showed a clear dominance of α-contraction were included in each data set (given that only 1 vessel displayed a clear β-dilation response at all adrenaline concentrations).

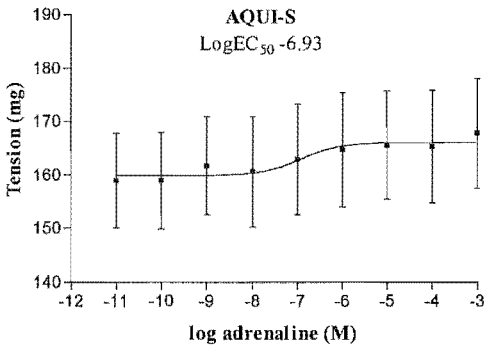
Dose response curves for AQUI-S and MS222 and treated vessels are shown in Figure 4.4. Values are means ± 1 SEM. Control values are also shown in Figure 4.4. The mean $LogEC_{50}$ in the presence of AQUI-S was –6.93 ± 0.418, (n=7); MS222, -6.17 ± 0.349 (n=8); and control vessels, -5.86 ± 0.148 (n=6). There was no significant difference between $LogEC_{50}$ values for each treatment.

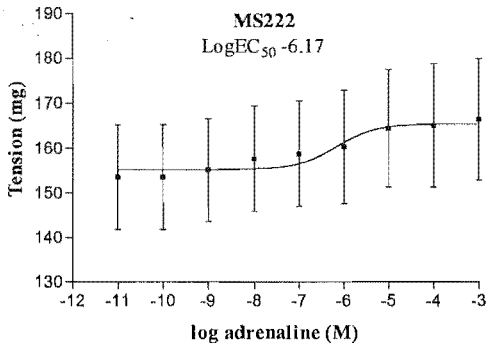
Series 4.2. Vasoactive effects of anaesthetics on the vasculature of Chinook salmon *in situ.*

Experiment 4.2.a. Vasoactive effects of AQUI-S on the vasculature of the perfused tail of Chinook Salmon.

Mean values for changes in pressure within the perfused tail throughout Experiment 4.2.a. can be seen in Figure 4.5. Because starting pressures varied between experiments, values are presented as % values of the maximum pressure within an experiment.

Exposure to all concentrations of AQUI-S elicited a significant and reversible decrease in pressure (vasodilation) in the perfused tail preparation (Figure 4.5). There was no significant difference between perfusion with 40ppm AQUI-S and 60ppm AQUI-S although pressures during perfusion with 80ppm AQUI-S were significantly lower ($P < 0.05$) than pressures during perfusion with 40ppm AQUI-S.





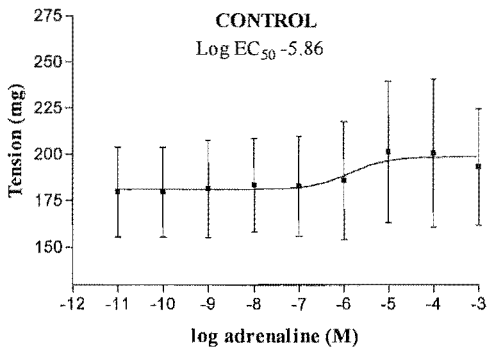


Figure 4.4.a-c. Adrenaline dose response curves for the Chinook salmon HPV in the presence of AQUI-S, MS222 or no anaesthetic. Data is presented as change in tension in milligrams (mg). All values are means ± 1 SEM.

Perfusion with 200ppm MS222 caused a decrease in pressure that was significantly greater ($P < 0.001$) than all concentrations of AQUI-S tested (Figure 4.5).

Perfusion with $1x10^{-5}$M adrenaline caused a significant increase ($P < 0.001$) in pressure compared to all other values throughout the experiment.

Experiment 4.2.b. Vasoactive effects of MS222 on the vasculature of the perfused tail of Chinook salmon.

Mean values for changes in pressure within the perfused tail preparation throughout Experiment 4.2.b. can be seen in Figure 4.6. Again, values are presented as % values of the maximum pressure within an experiment.

Exposure to all concentrations of MS222 elicited a significant and reversible decrease in pressure (vasodilation) in the perfused tail perfusion (Figure 4.6). Perfusion with 200ppm MS222 caused the greatest decrease in pressure and its effect was significantly ($P < 0.01$) different from 50ppm MS222 and 100ppm MS222.

There was no significant difference between the effect of MS222 on the tail vasculature versus the effect of AQUI-S.

Perfusion with $1x10^{-5}$M adrenaline caused a significant increase ($P < 0.001$) in pressure compared to all other values throughout the experiment.

Analysis of the effect of holding time on the response of the tail vasculature to anaesthetic.

The following section presents data for both anaesthetics from fish following division into 2 groups based on the length of time they were held before experimentation. The 2 groups are labelled 'new' fish (held for a short period) and 'old' fish (held for an extended period of time). These data are subsets of those recorded in section 4.2 above.

For further details of differences in holding times, refer to Series 4.2. in the methods and materials section.

Data are presented as a comparison between new and old fish for each anaesthetic. Statistical analysis of the effects of each anaesthetic was carried out as described for Series 4.2.

Comparison of the effect of AQUI-S anaesthetic on the tail vasculature of Chinook salmon; 'new' versus 'old' fish.

Mean values (± 1 SEM) for changes in pressure within the AQUI-S perfused tail for 'new' and 'old' fish can be seen in Figure 4.7. and Figure 4.8. respectively. Values are presented as % values of the maximum pressure within an experiment

'New' Fish.

Although on exposure to the anaesthetic, small decreases in pressure were apparent within the tail preparation, results were variable and changes in pressure on exposure to any concentration of AQUI-S tested were not significant (Figure 4.7.).

'Old' Fish.

In contrast to 'New' fish, tail preparations from 'Old' fish showed a significant ($P < 0.05$, $n=6$) and reversible drop in pressure compared to pre-exposure and recovery, on exposure to all concentrations of AQUI-S (Figure 4.8.). For all concentration of AQUI-S, this drop in pressure was significantly different ($P < 0.01$, $n=6$) from the effects of the same concentration of anaesthetic in 'new' fish.

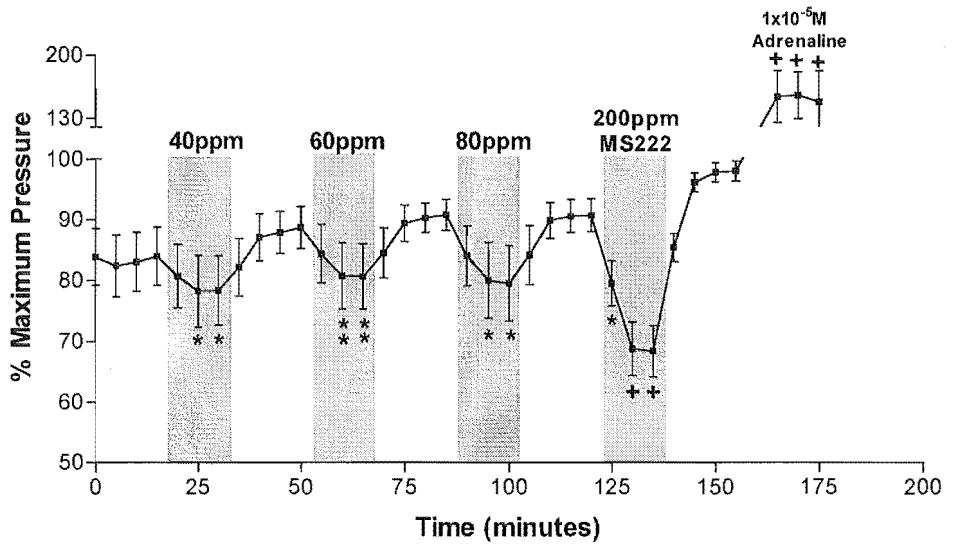


Figure 4.5. Mean values for pressure changes in the perfused tail of the Chinook salmon on exposure to increasing concentrations of AQUI-S anaesthetic. Exposure to anaesthetic (labelled shading) initiated a significant, reproducible and reversible fall in pressure within the preparation.
* denotes a significant difference from pre-exposure and recovery ($P < 0.05$, n=12).
** denotes a significant difference from pre-exposure and recovery ($P < 0.01$, n=12).
+ denotes a significant difference from pre-exposure and recovery ($P < 0.001$, n=12).

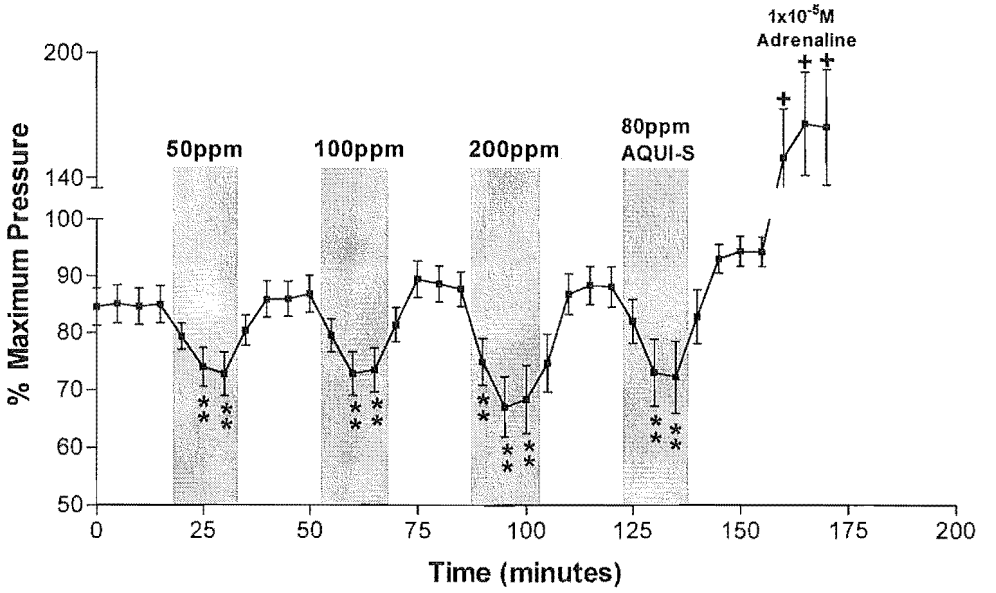


Figure 4.6. Mean values for pressure changes in the perfused tail of the Chinook salmon on exposure to increasing concentrations of MS222 anaesthetic. Exposure to anaesthetic (labelled shading) initiated a significant, reproducible and reversible fall in pressure within the preparation.
* denotes a significant difference from pre-exposure and recovery ($P < 0.05$, n=12).
** denotes a significant difference from pre-exposure and recovery ($P < 0.01$, n=12).
+ denotes a significant difference from pre-exposure and recovery ($P < 0.001$, n=12).

Comparison of the effect of MS222 anaesthetic on the tail vasculature of Chinook salmon; 'new' versus 'old' fish.

Mean values (± 1 SEM) for changes in pressure within the MS222 perfused tail for 'new' and 'old' fish can be seen in Figure 4.9 and Figure 4.10. respectively. Values are presented as % values of the maximum pressure within an experiment

'New' Fish.

In 'New' fish perfusion with 50ppm or 100ppm MS222 had no significant effect on pressure within the preparation, compared to pre-exposure. However, values during perfusion with both concentrations were significantly different ($P \leq 0.05$, n=5) from recovery values at 10-minutes post anaesthesia.

Perfusion with 200ppm MS222 caused a significant ($P \leq 0.05$, n=5) yet reversible decrease in pressure compared to both pre-exposure and recovery values.

Perfusion of the tail preparation with 80ppm AQUI-S in tails previously perfused with MS222, resulted in a significant ($P \leq 0.05$) vasodilation compared to 80ppm AQUI-S in fish only exposed to AQUI-S

'Old' Fish.

In 'Old' fish, all concentrations of MS222 tested elicited a significant ($P < 0.01$, n=6) and reversible (although not dose-dependant) drop in pressure within the perfused tail preparation (Figure 4.10.). For all concentrations, this drop in pressure was significantly greater ($P < 0.01$, n=6) than the effects of the same concentration of MS222 in so-called 'new' fish.

There was no significant difference between the effects of 200ppm MS222 in fish exposed only to MS222, versus the same anaesthetic concentration in fish previously exposed to AQUI-S.

Addition of $1x10^{-5}$M adrenaline to 3 of 6 MS222 treated preparations elicited a significant ($P < 0.05$, n=3) increase in pressure compared to all other times throughout the experiment. In the remaining 3 experiments, adrenaline elicited vasodilation within the preparation

Comparison of effects of AQUI-S and MS222 in 'New' Fish.

In 'New' fish, the effect of 50ppm MS222 was not significantly different from that of 40ppm AQUI-S. However, both 100ppm and 200ppm MS222 caused significantly larger ($P \leq 0.05$) falls in pressure within preparations than their matched concentrations of AQUI-S (Figure 4.9). This implies that overall, the tail vasculature was more responsive to MS222 than AQUI-S in 'New' fish.

Comparison of effects of AQUI-S and MS222 in 'Old' Fish.

In 'Old' fish, there was no significant difference between the effects of all concentrations of AQUI-S or MS222. Both anaesthetics elicited significant falls in pressure within tail preparations at all concentrations.

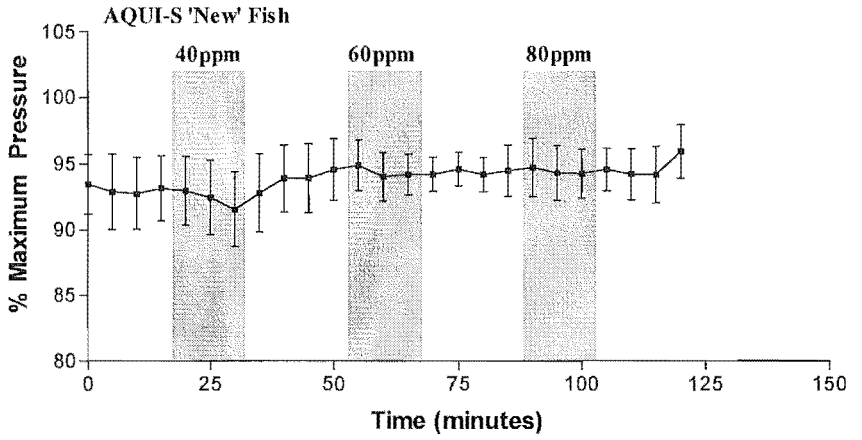


Figure 4.7. Mean values for pressure changes in the perfused tail preparation of Chinook salmon in 'new' fish perfused with AQUI-S. Shading indicates time of anaesthetic perfusion while label indicates concentration used at that specific time. There was no significant change in pressure due to perfusion with anaesthetic.

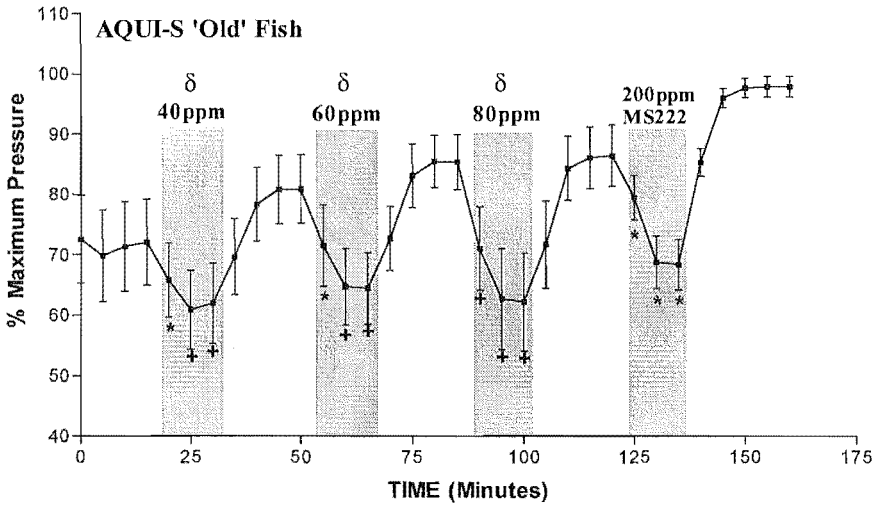


Figure 4.8. Mean values for pressure changes in the perfused tail preparation of Chinook salmon in 'Old' fish perfused with AQUI-S. Shading indicates time of anaesthetic perfusion while label indicates concentration used at that specific time. All concentrations of anaesthetic elicited a significant and reversible drop in pressure within the tail preparation.
*denotes a significant difference from pre-exposure and recovery ($P < 0.05$, n=6)
+ denotes a significant difference from pre-exposure and recovery ($P < 0.01$, n=6)
δ denotes a significant difference from the same anaesthetic concentration when compared to 'New' fish ($P < 0.01$, n=6).

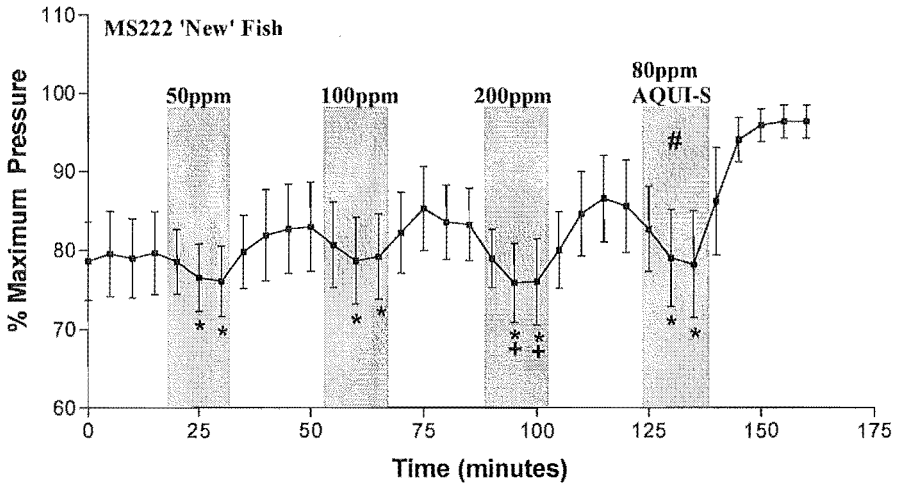


Figure 4.9. Mean values for pressure changes in the perfused tail preparation of Chinook salmon in 'new' fish perfused with MS222. Shading indicates time of anaesthetic perfusion while label indicates concentration used at that specific time. * represents a significant difference from the following 10 minutes post anaesthesia addition ($P \leq 0.05$, n=5). + represents a significant difference from pre-anaesthetic pressure values ($P \leq 0.05$, n=5).
represents a significant difference from the same anaesthetic treatment in fish exposed to AQUI-S ($P \leq 0.05$, n=5).

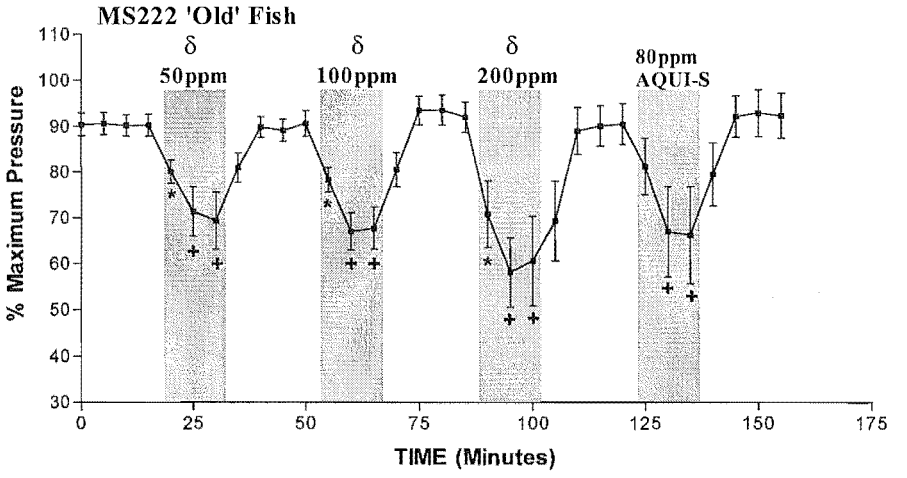


Figure 4.10. Mean values for pressure changes in the perfused tail preparation of Chinook salmon in 'Old' fish perfused with MS222. Shading indicates time of anaesthetic perfusion while label indicates concentration used at that specific time. All concentrations of anaesthetic elicited a significant and reversible drop in pressure within the tail preparation.
*denotes a significant difference from pre-exposure and recovery ($P < 0.05$, n=6)
+ denotes a significant difference from pre-exposure and recovery ($P < 0.01$, n=6)
δ denotes a significant difference from the same anaesthetic concentration when compared to 'New' fish ($P < 0.01$, n=6).

Discussion

Adrenaline dose response curves.

Anaesthetic agents are well known to affect the cardiovascular system, not only *via* actions on the sympathetic nervous system, but by direct chemical effects and effects on hormonal systems (Sellgren et al., 1992). Anaesthetics also have profound effects on pressor responses to various vasoactive drugs (Tabrizchi and Pang, 1992).

With this is mind, the response of Chinook salmon veins to adrenaline in the presence of either AQUI-S or MS222 anaesthetic was tested. $LogEC_{50}$ values obtained for adrenaline obtained in this study were marginally higher than previously reported values for arteries in other species of teleost fish. Values have been reported for cod coelic artery of –6.43 (Holmgren and Nilsson, 1974), perfused eel tail, -6.63 (Davie, 1981) and for rainbow trout coelic arteries and perfused splanchnic preparations of –7.96 (Holmgren and Nilsson, 1974) –7.66 (Xu and Olson, 1993(a)) and –6.38 (Xu and Olson, 1993(b)). The difference seen between salmon and trout is not wholly unexpected given that different vessels were used (arteries versus veins) and the fact that cod and trout coelic arteries exhibited differences in $LogEC_{50}$ values within a single study (Holmgren and Nilsson, 1974). Also, catecholamines can both constrict or dilate blood vessels depending on the adrenergic receptor (α or β) that is stimulated. In the current experiments, β antagonists were not used to prevent adrenaline-mediated dilation of the vessels. This meant that the catecholamines administered could bind to both receptor types. $LogEC_{50}$ values for adrenaline in the presence of either AQUI-S or MS222 were no different indicating a lack of effect of anaesthetics on the ability of blood vessels to respond to physiological and supraphysiological doses of adrenaline. Nor does pre-exposure to high concentrations of AQUI-S or MS222 appear to affect the vascular response to adrenaline in perfused salmon tail preparations. Thus MS222 and AQUI-S do not appear to affect the functioning of the α-adrenergic receptors effecting vasoconstriction.

Given that catecholamines are the primary hormonal regulators of cardiovascular function during periods of extreme stress in fish (Randall and Perry, 1992), any drug that modifies the 'normal' *in vivo* response to catecholamines could cause increased mortalities.

In both the isolated vessel myography and perfused tail experiments, exposure to either AQUI-S or MS222 anaesthetic caused a significant vasodilation of blood vessels.

Both these anaesthetics at the concentrations tested in this study, have been shown to cause dilation of afferent and efferent branchial arteries in the Chinook salmon and the spotty (*Notolabrus celidotus*) (Hill, 1999). MS222 also causes dilation of the secondary lamellae in rainbow trout (Soivio and Hughes, 1978). In both fish and mammals, anaesthetic induction reduces systemic blood pressure (present study; Hill, 1999; Sellgren et al., 1992) and local anaesthetics are often administered in combination with adrenaline to improve their efficacy (Meechan, 1990). The dilatory actions of anaesthetics are most likely due in part to a direct effect on the smooth muscle cell and in part to their inhibitory effects on the sympathetic nervous system. Sympathetic neurons contribute to blood pressure homeostasis by regulating vascular resistance (Baum, 1977; Sellgren et al., 1992). As discussed in Chapter 2, anaesthetics act via widespread depression of the central nervous system. Although the exact mechanisms of anaesthetic action are still not fully understood, anaesthetics appear to have direct effects on a variety of ion channels in both mammals and teleosts, including Na^+, Ca^{2+} K^+ and $GABA_A$ channels (Meechan, 1990; Sellgren et al., 1992; Quast, 1993; Wartenberg et al., 1994; Benoit, 1995). Anaesthetics also appear to have direct effects on glutamate receptors, 5HT receptors and nicotinic acetylcholine receptors (Ragsdale et al., 1994; Benoit, 1995; Tonner and Miller, 1995; Trudell and Bertaccini, 2002; Ingvaast Larson, 2003).

Contraction of vascular smooth muscle is a Ca^{2+} dependant process. Smooth muscle contraction is activated either by an increase in myoplasmic free Ca^{2+} or a change in the sensitivity of the contractile apparatus for Ca^{2+} (Quast, 1993; Rűeg, 1998; Hill et al., 2001). Contractile agonists such as adrenaline initiate phospholipase C hydrolysis of PIP_2 to IP_3 and DAG (Quast, 1993). IP_3 goes on to release Ca^{2+} from intracellular stores while DAG acts with the released Ca^{2+} to activate protein kinase C, which sensitises the contractile apparatus (Quast, 1993).

In hypertensive rats, isoeugenodilol, a chemical derivative of isoeugenol (the active component of AQUI-S) has been shown to decrease mean blood pressure and heart rate in a dose dependant manner (Yeh et al., 2000). This vasorelaxant activity was attributed to ability of isoeugenodilol to inhibit L-type Ca^{2+} channels, receptor mediated Ca^{2+} mobilisation and stimulation of K^+ channel opening (Yeh et al., 2000). This idea is supported by recent work investigating the mode of action of eugenol (the active ingredient in clove oil) on rings of rat thoracic aorta (Damiani et al., 2003). In isolated aortic rings, eugenol produces a concentration dependant depressant effect on KCl and phenylephrine induced contractions. The inhibitory effects of eugenol were attenuated, but not prevented by removal of the endothelial layer. This result, coupled with the fact that

eugenol also reduced the contraction resulting from α_1-adrenoceptor stimulation suggests that eugenol might interfere with both voltage and receptor operated Ca^{2+} channels, reducing Ca^{2+} influx and consequently contraction (Damiani et al., 2003). Although the maximal vasorelaxing actions of eugenol were not inhibited in the presence of the Nitric Oxide blocker, L-NAME, or guanylyl cyclase inhibitor, methylene blue, sensitivity to eugenol was greatly reduced. This suggests that the actions of eugenol may be partially endothelium-dependent and mediated by the Nitric-oxide- guanylyl cyclase pathway. The authors suggest that endothelial dependence is seen at low concentrations (300μM) of eugenol, whereas at higher concentrations (10^{-6}-10^{-2}M), the Ca^{2+} channel blocker action prevails (Damiani et al., 2003). In regards to AQUI-S specifically, recent work has indicated that isoeugenol causes neuro muscular blockade in rat phrenic nerve diaphragm preparations, acting as a competitive non-depolarising blocker of nicotinic acetylcholine receptors on the muscle end plate (Ingvast-Larsson et al., 2003). These studies indicate that AQUI-S, through its active component isoeugenol, can have a direct effect on the contractile ability of the vascular smooth muscle and inhibit adrenergically mediated smooth muscle contraction, at least in rats. Further work would be required to elucidate the mechanism of vasorelaxation during AQUI-S anaesthesia in salmon.

Effects of vasorelaxation *in vivo*.

What might this vasorelaxant effect of anaesthetics mean for the anaesthetised animal? Basal vascular tone is set by myogenic activity of the smooth muscle cells of the vascular system, which contract to counteract the stretch caused by blood pressure (Bushnell, 1992). In fish, the circulation is a single pass system where the branchial and systemic vascular beds represent the 2 major sites of resistance (Satchell, 1991). A generalised picture of this circuit is as follows: the heart generates cardiac output that enters the gills via the ventral aorta and the afferent branchial arteries, crosses the lamellae and exits via the efferent branchial arteries. The dorsal aorta transports the oxygenated blood around the body differentiating into a complex series of arterioles, capillaries and finally veins. Peripheral veins empty into central veins that return blood to the heart where the circuit begins again (Satchell, 1991; Bushnell, 1992; Olson, 1998b). Blood flow through peripheral vessels is primarily regulated by vasomotor nerves to the trunk. Venous return is affected by several factors including arteriolar resistance and tone and compliance of the systemic veins (Satchell, 1991; Olson, 1998b). If arteriolar resistance increases, blood flow through the capillaries and veins is reduced decreasing the

transmural pressure and the volume of blood contained in the veins hence decreasing venous return to the heart (Satchell, 1991).

Anaesthetic induced dilation of the arterioles will theoretically have the opposite effect. Blood flow and hence volume of blood to the capillaries and veins will increase due to the decrease in resistance. Cardiac output is a product of heart rate and stroke volume. In turn, stroke volume is determined by ventricular end diastolic volume and myocardial contractility (Olson, 1998b). A reduction in either of these factors due to anaesthesia will cause a concomitant decrease in cardiac output. In perfused rainbow trout heart preparations, MS222 reduced ventricle contraction by up to 25% and contractile force of ventricle strips fell dose dependently with increasing concentrations of MS222 (Ryan et al., 1993). A reduction in cardiac output during anaesthesia will reduce the amount of blood perfusing the gills, even though the gill vasculature may also dilate in response to anaesthetic (Soivio and Hughes, 1978; Hill, 1999).

In non-anaesthetised fish, arterial hypotension tends to decrease heart rate and reflexively increase central venous pressure and systemic pressure in the trout (Wood and Shelton, 1980, cited in Olson, 1998a). It is possible that this is mediated by baroreceptor activity (Olson, 1998a). In humans, anaesthetic agents have been shown to inhibit skeletal muscle nerve sympathetic activity as well as significantly inhibiting baroreceptor mediated reflexes (Sellgren et al., 1992). *In vivo*, (refer Chapter 2) heart rate increased significantly in salmon during anaesthesia with AQUI-S. It is possible that this rise in heart rate was due to a combination of vagal release and also a reflex mediated attempt to offset the drop in dorsal aortic pressure seen during induction.

Effects of vasodilation on venous return

Anaesthetic induced vasodilation may also have an effect on venous return. The post-abdominal tail represents a specialised region for facilitating venous return (Satchell, 1991). In the post abdominal region, the ventral processes of the spine fuse, forming the haemal arch which encloses the dorsal aorta and caudal vein. Segmental arteries arise from the dorsal aorta to supply capillaries of the white and red muscle (Satchell, 1991). Blood from the red and white muscles is collected into the segmental veins, which then drain into the caudal vein. Under normal conditions, swimming movements compress the muscle blocks and this action 'pumps' blood from the segmental veins into the caudal vein and towards the heart (Satchell, 1991). Ostial valves at the opening into the caudal vein prevent backflow of blood back into the segmental veins during swimming. Venous

pressures in the tail are generally very low in teleost fish (Bushnell, 1992; Satchell, 1991) and the haemal pump in the tail enhances venous return during swimming.

If anaesthesia reduces mean venous pressure by inducing a vasodilation of the tail veins, then blood return to the heart may be reduced. Decreased muscle tone due to neuromuscular blockade might also reduce the proportion of venous return attributed to the action of the haemal pump.

However, a vasodilator effect on the systemic vasculature may also counteract cardiovascular stresses associated with a high haematocrit. Blood viscosity is related to the fraction of red blood cells in the blood and thus systemic resistance is related to haematocrit (Bushnell et al., 1992). The heart must generate enough force to overcome vascular resistance. An increase in haematocrit increases systemic resistance and concomitantly the amount of work required by the heart to overcome this resistance. Dilation of the systemic vessels may offset the increases in haematocrit seen during hypoxia and anaesthesia, thereby reducing energy demands on the heart. However, it may also offset the benefit derived from an increase in blood capacitance for oxygen.

With regard to the tail perfusion experiments in Series 4.2 it was seen that for both MS222 and AQUI-S, dilation of the tail vasculature in response to anaesthetic was attenuated in fish held for less than 2 weeks prior to experimentation. This group of fish were labelled 'new' fish. It is not known why these results occurred, but the reduced vasoactivity of AQUI-S in this group was highly reproducible. The obvious question is therefore, how do the 'new' fish differ physiologically from 'old' fish? The answer, at this point, can only be guessed at. However, a possible reason is offered below.

It is likely that the fish held for a shorter time were experiencing a stress response due to the relatively short time for aquarium acclimation. This is generally characterised by high levels of plasma catecholamines and cortisol (Reid and Perry, 1992; Mommsen et al., 1999). Both these hormone families affect teleost metabolism and responsiveness of various physiological systems to different stimuli (Reid and Perry, 1991; Perry et al., 1996; Perry and Bernier, 1999; Mommsen et al., 1999).

Proposed models of anaesthetic action.

AQUI-S has been shown to affect nicotinic acetylcholine receptors in the muscle end plate of rats (Ingvast-Larsson, 2003) causing smooth muscle relaxation. The acetylcholine receptor exists in several different states; resting, open and desensitised (Tonner and Miller 1995; Shipton, 1999). Various anaesthetics of different chemical

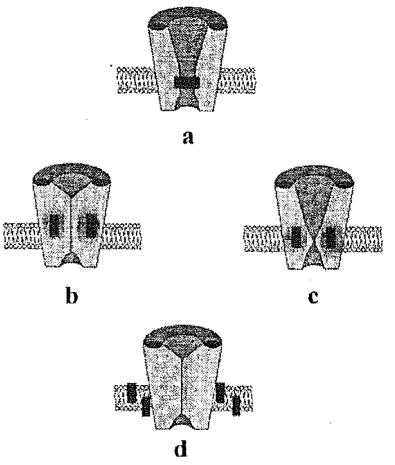


Figure 4.11 Proposed mechanisms of anaesthetic action on nicotinic acetylcholine receptors. (a) Inhibition of receptor function by binding of the anaesthetic to a site within the channel lumen. (b) and (c) Allosteric inhibition of the receptors by anaesthetic binding to a site remote from the channel preventing channel opening. (d) Inhibition of the receptor by anaesthetic induced changes of the lipid bi-layer and/or lipid protein interface. (Taken from Tonner and Miller, 1995).

composition interact with different receptor sites exerting both specific and non-specific actions on the receptor (Tonner and Miller, 1995). Several models of anaesthetic interaction with nicotinic acetylcholine receptors and other membrane ion channels have been proposed (Tonner and Miller, 1995; Benoit, 1995; Shipton, 1999).

a) Direct inhibition or the receptor ion channel by binding of the anaesthetic to a site within the channel lumen; (Figure 4.11, (a)).

b) Binding to an allosteric site on the receptor, which changes the receptor conformation thereby preventing channel opening; (Figure 4.11, (b & c)).

c) Inhibition of the receptor by anaesthetic induced changes of the lipid bi-layer and /or the lipid protein interface (Figure 4.11, (d)).

If 'new' fish had raised plasma catecholamine and/or cortisol levels and if these hormones in some way modified, or affected proteins in the lipid bi-layer associated with the nicotinic acetylcholine receptor, it is possible that their action inhibited AQUI-S stimulated vasodilation. During stress, cortisol has been shown to be important in elevating mean blood pressure via central nervous system stimulation (Bamberger et al., 1996, cited in Mommsen et al., 1999). Davidson et al., (2000) demonstrated an increase in

plasma cortisol levels upon AQUI-S anaesthesia. However, in the present study fish were not exposed to AQUI-S until it was added to the perfusion medium.

In the so-called 'old' fish, levels of cortisol may have been reduced to concentrations that no longer inhibited the proposed mechanism of AQUI-S induced vasodilation. Given the vasodilatory effect of MS222 on the tail vasculature of both 'new' and 'old' fish, it is likely that this anaesthetic acts more directly on specific ion channels. MS222 is used as a local anaesthetic in mammals. Following injection, the anaesthetic dissociates into a lipid soluble free base, which passes through the cell membrane into the nerve axon. Here, it is reionised and enters the Na^+ channels, effectively 'plugging' them (Aitkenhead and Smith, 1996). In the squid axon, MS222 blocks signal conductance by suppressing Na^+ movement across the membrane (Frazier and Narahashi, 1975). It is unlikely Na^+ channels on the axon membrane would be altered during stress, and therefore this may explain why MS222 elicited vasodilation in both groups of fish.

Obviously to elucidate possible reasons for these differences requires further experimental work. The effects of artificially raised cortisol and/or catecholamines on the response of both 'new' and 'old' fish could be investigated. This would however be intensive and time-consuming and would best suit a study aimed at elucidating the mechanism of AQUI-S anaesthesia in fish. Recent work in the rat has indicated that AQUI-S, although causing neuromuscular blockade may not inhibit sensory neurons (Ingvast-Larsson, 2003). Therefore this could be an interesting and very important dilemma to resolve.

In conclusion, in both mammals and teleosts, anaesthetics are known to cause a decrease in blood pressure *in vivo*. This is most likely mediated by the anaesthetic effects on the sympathetic nervous system and via direct effects on Ca^{2+} movements in the vascular smooth muscle. This drop in systemic pressure, coupled with possible direct effects of anaesthetics on the myocardium could cause significant stress on the heart as it attempts to maintain blood flow. However, anaesthetic induced vasodilation may also reduce workload on the heart and offset hypoxia initiated increases in haematocrit.

Chapter 5

General discussion.

The exact molecular mechanisms of anaesthetic actions still remain unclear despite extensive research and debate on the subject. However, it is agreed that anaesthetics, both general and local exhibit profound effects on the cardiovascular system of vertebrates (Black, 1980; Quast, 1993; Hill, 1999).

In Chinook salmon, it was seen that light anaesthesia with AQUI-S caused a decrease in blood pressure coupled with an increase in heart rate from resting values. During recovery, blood pressure rose and heart rate fell to resting values. However, there was no significant increase in circulating plasma catecholamines, suggesting that anaesthetic action alone is not responsible for increases in plasma catecholamines seen in previous studies (Iwama et al., 1989; Hill, 1999). This was corroborated by *in situ* perfusion of the salmon chromaffin tissue with AQUI-S concentrations high enough to induce anaesthesia in the whole animal. AQUI-S did not stimulate catecholamine release from the chromaffin tissue. *In vivo*, there were marked changes in haematocrit and mean cell haemoglobin concentrations consistent with well-established red blood cell responses to hypoxia. Theses effects were reproducible *in vitro*, when red blood cells were exposed to hypoxic conditions. However, hypoxia alone did not stimulate catecholamine release from the chromaffin tissue of the salmon, *in situ*.

During prolonged anaesthesia and surgery, both Chinook salmon and Snapper released high concentrations of both adrenaline and noradrenaline into the circulation. In this case however, changes seen in haematological variables are most likely reflective of adrenergically mediated red blood cell responses combined with a direct response to hypoxia.

Given the changes in blood pressure seen during anaesthesia *in vivo*, a series of *in vitro* and *in situ* experiments using isolated rings of hepatic portal vein and perfused tail preparations, were developed to investigate whether anaesthetics could exert direct effects on the vascular smooth muscle. Both AQUI-S and MS222 caused a significant vasodilation of vascular smooth muscle in the hepatic portal vein and the tail vasculature of the Chinook salmon.

From these results, several conclusions can be made.

- Anaesthetic induced hypoxia is the proximate stimulus for catecholamine release
- Hypoxaemia at the level of the chromaffin tissue, in the absence of other stimuli, is not sufficient to stimulate catecholamine release.
- AQUI-S and MS222, in low concentrations, appear to have little effect on the ability of Chinook veins to react to catecholamines, *in vitro*.
- High concentrations of catecholamines are cleared from the circulation of both Chinook salmon and Snapper rapidly following recovery from anaesthesia.
- AQUI-S and MS222 appear to have direct effects on the vascular smooth muscle of the Chinook salmon tail and on hepatic portal veins.

We can also construct an image of what may happen as a fish undergoes general anaesthesia. As the fish is exposed to the anaesthetic (e.g 60ppm AQUI-S), the initial response seen is as a decrease in P_{DA} possibly due to a direct effect of the anaesthetic on the vascular smooth muscle. Heart rate begins to reflexively increase to offset this. As anaesthesia deepens, vagal inhibition on the heart is reduced and heart rate continues to increase steadily. The red blood cells begin to swell, possibly due an increase in plasma pH. Accumulation of anaesthetic in neural tissues reduces ventilation and may begin to have a direct inhibitory effect on the myocardium of the heart. A state of severe hypoxia now ensues. As blood PO_2 falls to around 50% saturation, catecholamines are released from the chromaffin tissue into the circulation. These catecholamines act to maintain blood pressure in the face of increasing anaesthetic load. Possible β-adrenergic action on the heart helps to maintain heart rate. The activation of Na^+/H^+ exchangers on the red blood cell membrane and the recruitment of red blood cells from the spleen maintain Hb-O_2 affinity and blood oxygen saturation. Upon recovery, circulating plasma catecholamines are quickly sequestered by the organs and metabolised/excreted by the gill. Table 5.1 gives a comparison between the cardiovascular changes seen during hypoxia and those seen during anaesthesia, based on the observations of the present study.

Actions of Catecholamines.

The catecholamine hormone system is one of the most important endocrine systems that has evolved in vertebrates, due primarily to its extensive and powerful

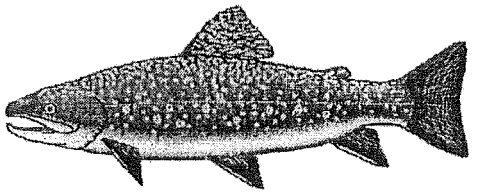
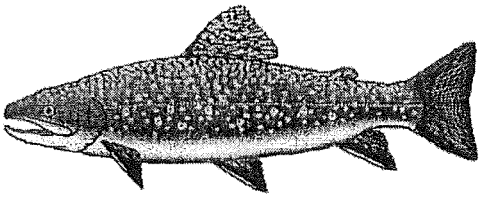
HYPOXIA	ANAESTHESIA
	
▪ Ventilation ⇑	▪ Ventilation ⇓
▪ P_{DA} ⇑	▪ P_{DA} ⇓
▪ Heart Rate ⇓	▪ Heart Rate ⇑
▪ Hypoxia induced RBC swelling	▪ Hypoxia induced RBC swelling
▪ Catecholamine release ~blood P_{50}	▪ Catecholamine release ~blood P_{50}
▪ Catecholamine induced RBC swelling and RBC recruitment from spleen	▪ Catecholamine induced RBC swelling and RBC recruitment from spleen

Table 5.1 Comparison of *in vivo* cardiovascular changes seen during hypoxia and anaesthesia.

interaction with the cardiovascular system. The catecholamine stress response in vertebrates has evolved through natural selection pressure to respond to those situations which present the greatest challenges for a particular animal. Not all vertebrates will release similar concentrations of catecholamines under the same environmental stress. For example, humans are known to release significant plasma concentrations of adrenaline in response to hypovolemia and intense mental stimulation i.e problem solving (Esler et al., 1990). Although catecholamine release is a general response to hypovolemia in vertebrate groups, it is not clear whether fish for example, would have need of an adaptive response to problem solving. Fish are more likely within their lives to encounter periods of extreme hypoxia and therefore this is a strong trigger for catecholamine release in many teleost fish. It is unlikely that humans routinely encountered severe hypoxia throughout their evolution and consequently hypoxia alone is not a strong stimulator of catecholamine release.

In the resting state, catecholamines are important neurotransmitters released from adrenergic nerve terminals. Fish blood vessels are generally well innervated, and the basal vascular tone is maintained in part by neuronal adrenergic influence (Olson, 1998b; Hill et al., 2001). Plasma catecholamines appear to have little or no influence on vascular tone during rest (Conklin and Olson, 1994) although circulating catecholamines may be an

important pool of neuronal catecholamine uptake, possibly sustaining neuronal sympathetic tone (Xu and Olson, 1993a) Direct effects on systemic vascular resistance only occur following an acute stress when they are released into the circulation in high concentrations e.g.>200nM (Randall and Perry, 1992; Wendelaar Bonga, 1997; Perry and Bernier, 1999).

Despite the long-held view that release of high concentrations of plasma catecholamines is a general feature of the primary stress response in fish, there is growing evidence to suggest that catecholamines are only released into the circulation in high concentrations during intense or chronic stress (Randall and Perry, 1992; Perry and Bernier, 1999; Perry et al., 2004). These stresses include severe hypoxia, exhaustive exercise and hypercapnia, all of which seriously compromise blood oxygenation (Boutilier et al., 1986; Aota et al., 1990; Randall and Perry, 1992: Perry et al., 2004). The pivotal role of plasma catecholamines during acute stress is undisputed and their physiological importance is illustrated by the wide variety of neuroactive peptides that trigger their release from the chromaffin tissue (refer Chapter 3). These alternate pathways of catecholamine release may represent possible 'backup' systems that came into play when nicotinic pathways are blocked or inhibited in some way.

Stimulation of catecholamine release

One of the central questions in terms of the catecholamine mediated stress response in fish, has focussed on the proximate stimulus for catecholamine release from the chromaffin cells. Studies over the past decade have emphasised the relationship between a decrease in blood oxygen-haemoglobin affinity and catecholamine release. In several teleost species, it has been shown that during hypoxia catecholamine release occurs abruptly at a critical PaO_2 value corresponding to the P_{50} value of blood (for references see Randall and Perry, 1992; Perry and Bernier, 1999; Perry et al., 2004). Although rainbow trout acclimated to varying temperatures (Perry and Reid 1994, cited in Perry and Bernier, 1999) or hypercapnia (Julio et al., 1998) exposed to hypoxia released adrenaline into the circulation at differing PaO_2 values compared to control fish, this value was correlated to their respective P_{50} values. This response has also been observed in the tropical fish ..... and ..... (Perry et al., 2004). When the obligate air breather ..... was exposed to hypoxic water (PO_2 <10 Torr) but allowed access to air, it did not release catecholamines. However, when th experiment was repeasted and the fish not allowed access to air, catecholamine were relased into the circulation when bool oxygen saturation fell below P_{50}. Thus it appears that a depression of blood oxygen content rather than a

decrease in PO_2 is the proximate stimulus for catecholamine release in teleost fish. The ability of catecholamines to increase blood Hb-O_2 biding affinity has been discussed in detail previously (refer Chapter 3, discussion).

In trout, CO_2 excretion from the red blood cell is dependant on the release of Bohr protons from haemoglobin during oxygenation (refer Chapter 3 for details). Oxygenation of trout blood *in vitro* is non-linear with the majority of Bohr protons being released between 60–100% Hb saturation (Brauner et al., 1996b). Oxygenation of blood over this area of the Hb-O_2 equilibrium curve also enhanced HCO_3^- flux rate across the HCO_3^-/Cl^- exchanger on the red blood cell membrane by 30%, whereas oxygenation below this point on the curve did not enhance CO_2 excretion *in vitro* (Brauner et al., 1996b). It is possible that the sudden reduction in red blood cell CO_2 excretion at values below 60% oxygen saturation is involved somehow in the stimulation of catecholamine release below blood P_{50} values in trout. A decrease in CO_2 excretion would tend to decrease red blood cell pH_i and decrease Hb-O_2 affinity. This drop in blood oxygenation and decreased pH_i stimulates catecholamine release, which in turn enhances Hb-O_2 affinity by raising pH_i.

How might hypoxia be sensed *in vivo*, and how does this stimulate catecholamine release from the chromaffin cells? There is recent evidence suggesting that humoral catecholamine secretion during hypoxia is mediated by peripheral O_2 chemoreceptors in trout gills (Reid and Perry, 2003). The role of O_2 chemoreceptors in stimulating catecholamine release in fish has been previously discussed in Chapter 3. O_2 chemoreceptors have been identified in the gill filaments of fish, and these cells resemble the chemoreceptor cells of mammals (Burleson and Milsom 1993). Although it seems likely that O_2 chemoreceptors in fish gills share similar transduction mechanisms as their mammalian counterparts due to their sensitivity to NaCN, the cellular mechanisms by which O_2 sensing is achieved are not fully understood (Burleson and Milsom, 1993; Reid and Perry, 2003). Studies of mammalian chemoreceptors have indicated a possible role of either K^+ or Ca^{2+} in stimulating neurotransmitter release from these chemoreceptors (Burleson and Milsom, 1993). Despite the lack of knowledge concerning the mechanisms of O_2 sensing, it is well known that these gill chemoreceptors respond to hypoxia (Reid and Perry, 2003). Via some as yet undescribed mechanism, O_2 chemoreceptors at the gill are stimulated during hypoxia, most likely by a decrease in blood PO_2. Somehow, this signal is conducted through the body to the chromaffin cells, which respond by releasing catecholamines into the circulation. There is no evidence as yet to suggest that anaesthetics have any direct inhibitory effects any proposed signal transduction system of

O_2 chemoreceptors. However, addition of propranolol, a β-adrenergic antagonist has been shown to attenuate the afferent response to hypoxia and NaCN in the trout gill. This was attributed to the membrane stabilising (anaesthetic) effect of propranolol rather than its antagonistic properties (Burleson and Milsom 1990, cited in Burleson and Milsom, 1993). However, in the present study, both salmon and snapper released high concentrations of plasma catecholamines. This suggests that AQUI-S at least does not modify chemoreceptor stimulated catecholamine release.

Metabolic actions of catecholamines.

The metabolic actions of catecholamines are summarised in Table 5.2. Catecholamines also act as glucoregulatory hormones, affecting carbohydrate and lipid metabolism both in fish and in mammals, although specifics vary between the two groups (Esler et al., 1990; Fabbri et al., 1998). Hypercapnia and hypoxia depress glycogen phosphorylase activity but increase pyruvate kinase activity, leading to a decrease in glucose levels (Randall and Perry, 1992). Following release into the circulation, catecholamines act rapidly to induce hyperglycemia, in part by activating hepatic glycogen phosphorylase and thereby stimulating glycogenolysis and glucogenolysis (Wendelaar Bonga, 1997; Fabbri et al., 1998; Perry and Bernier 1999). Adrenergic activation of a hormone sensitive triacylglycerol lipase results in liberation of glycerol and free fatty acids (Fabbri et al., 1998) although there are significant inter and intra species differences in the sensitivity of the tissues to catecholamine induced lipolysis (Fabbri et al., 1998). Catecholamine also sustain oxidative metabolism in trout red blood cells during acidosis in part by enhancing erythrocyte lactate oxidation (Wood et al., 1990, cited in Randall and Perry, 1992).

In marine teleosts, such as the snapper, catecholamines may also enhance H^+ extrusion across the gill epithelia, a process mediated by β-activation of Na^+/H^+ and Na^+/NH_4^+ exchangers (Claiborne, 1998). Adrenaline may also play a role in protecting the myocardium from acidosis. Accumulated protons during acidosis may compete with Ca^{2+} for binding sites on the myocardium, decreasing contractile force and cardiac output (Satchell, 1991). *In vitro* studies have indicated that this can be reversed by addition of physiological concentrations of adrenaline to perfused heart preparations, although the exact mechanism is unclear (Satchell, 1991).

Catecholamines are not the only hormones secreted during acute stress. The glucocorticoid cortisol is the major hormone released via the hypothalamic-Pituitary-Interrenal axis, and is primarily described in literature as a stress hormone (Wendelaar

Catecholamines	Cortisol
▪ Blood glucose ⇑	▪ Protein mobilisation
▪ Blood lactate ⇑	▪ Protein synthesis ⇑
▪ Changes in free fatty acids	▪ Inhibition of growth
▪ Tachycardia	▪ Carbohydrate utilisation ⇓
▪ Increased cardiac output	▪ ⇑ glucose production from tissue protein
▪ Vasodilation or vasoconstriction	▪ Glycogen deposition in the liver
▪ Increased Haematocrit	▪ Changes in membrane permeability
▪ Glucogenesis in the liver	▪ ⇑ production/activity of Na^+/K^+-ATPase

Table 5.2 The effects of catecholamine and cortisol release on the cardiovascular system and metabolism of fishes (Taken from Ross and Ross, 1984).

Bonga, 1997; Mommsen et al., 1999). Although cortisol is well documented as having similar effects as catecholamines on fish metabolism (Table 5.2), cortisol tends to have persistent, long-term effects remaining in the plasma in high concentrations for extended periods of time (Mommsen et al., 1999).

Anaesthesia and Hypoxia.

The actions of catecholamines and cortisol during either acute or prolonged stress have adapted fish to cope specifically with environmental stresses that would occur throughout their natural life. There is no natural selection pressure that would adapt fish specifically to anaesthesia, which presents a unique challenge to fish due to both direct chemical effects and central effects (i.e induction of hypoxia) on the cardiovascular system. Therefore, the fish has to respond to those anaesthesia-induced stressors for which it has specific physiological adaptive mechanisms, such as hypoxia.

As discussed in Chapter 2, a typical teleost response to hypoxia is characterised by increased ventilation, reflex bradycardia and an increase in systemic resistance (Satchell, 1991). In trout, the pulse pressure in both the ventral and dorsal aorta has been shown to increase during hypoxia coupled with an increase in resistance to blood flow at the gills (Holeton and Randall, 1967; Satchell, 1991). This may be achieved by catecholamine mediated actions on both the afferent and efferent branchial arteries. β-dilation of the afferent arterioles coupled with constriction of a muscular sphincter at the opening of the efferent arterioles increases the amount of blood perfusing the gill, physically increasing the surface area for gas exchange (Satchell, 1991) In rainbow and steelhead trout, the efferent arteries themselves have been shown to dilate in direct response to hypoxia (Smith et al., 2001). A similar effect on gill resistance has also been

seen in the atlantic cod, (Fritsche and Nilsson, 1990) and the pacific Hagfish *(Eptatretus cirrhatus)* (Forster et al., 1992). An increase in vascular resistance at the gills may be associated with shunting of the blood though alternate vascular pathways, increasing the blood sheet across the gills and decreasing the diffusion distance between blood and water (Holeton and Randall, 1967; Satchell, 1991) Gill perfusion during hypoxia is also enhanced by the maintenance of cardiac output, due almost entirely to an increase in stroke volume, achieved by increased filling times during bradycardia (Holeton and Randall, 1967; Fritsche and Nilsson, 1989; Satchell, 1991).

In the pacific hagfish, hypoxia increased branchial resistance although there was no change in heart rate or systemic vascular resistance even though cardiac output increased by 40% (Forster et al., 1992). A comparative study of three marine teleosts showed that individual species may exhibit some or none of the above described responses (refer Chapter 2; Fritsche, 1990). Differences between species may be indicative of ecological and behavioural differences. Vascular changes seen during hypoxia can be attributed to direct actions of high concentrations of circulating catecholamines.

Possible vascular effects of AQUI-S and MS222.

Any attenuation of the response to hypoxia due to either AQUI-S or MS222 would be most likely to occur in the vascular system, given the strong anaesthetic induced vasorelaxation seen both *in vitro* and *in situ*. Neither of these anaesthetics however appears to have significant effects on the ability of blood vessels to respond to adrenaline *in vitro*, although whether this represents the situation *in vivo* is unknown. If either anaesthetic blocks the actual contractile mechanisms of the smooth muscle (i.e ion currents), then one would assume that the ability of blood vessels to contract in response to adrenaline would be inhibited. However, this effect was not seen *in vitro* suggesting that anaesthetics may block neuronal adrenergic tone but have a lesser effect on the actions of plasma catecholamines.

In vitro AQUI-S and MS222 caused dilation of the HPV within 1 minute of anaesthetic addition. An almost identical time frame was seen in the perfused salmon tail ($\leq$ 1.5 minutes). *In vivo*, a decrease in P_{DA} was seen within 2 minutes of the start of induction with 60ppm AQUI-S. However, following 5 minutes of exposure to 60ppm AQUI-S, Chinook salmon showed no increase in circulating levels of plasma catecholamines. From these results it appears that *in vivo* the fish would likely experience a significant anaesthetic induced vasodilation well before catecholamines are released into the circulation. The effects of a systemic vasodilation have been discussed previously in

Chapter 4. Dilation of systemic vessels can decrease venous return due to pooling of the blood in peripheral tissues and a decrease in the efficiency of physical venous return mechanisms such as the haemal arch pump, which relies on the contraction of post-abdominal muscle blocks to move blood back towards the heart (Satchell, 1991).

If all blood vessels dilate in response to AQUI-S or MS222 to the same degree, then the overall effect on cardiovascular homeostasis may be relatively small. However, this is unlikely to be the case. Different vascular beds receive varying degrees of adrenergic and cholinergic innervation (Satchell, 1991; Olson, 1998b; Hill et al., 2001) and therefore may be differentially susceptible to various anaesthetics. This would obviously depend on the mode of action of the anaesthetic in question. Hill (1999) found that efferent branchial arteries from both spotty (*Notolabrus celidotus*) and Chinook salmon showed a greater dilation than afferent branchial arteries in response to either MS222 or AQUI-S. Whether these differing responses are 'good' or 'bad', would depend on the response of various vessels in the absence of anaesthetic. Normally the afferent pathways dilate during hypoxia, so an anaesthetic induced vasodilation might even enhance this response, moving more blood into the gill. However, as discussed above, the muscular sphincter at the opening to the efferent branchial arteries constricts during hypoxia, enhancing blood oxygenation at the gill. Dilation of the sphincter may favour the movement of blood through the marginal and basal channels and decrease the number of lamellae perfused (Soivio and Hughes, 1978; Satchell, 1991) thereby reducing blood oxygenation at the gill. It is likely that any anaesthetic induced changes in vessel resistance at the gill will occur prior to any systemic changes in resistance, given that the gill is the primary site of anaesthetic uptake, and will 'see' the anaesthetic directly.

While anaesthesia may counteract some of the actions of catecholamines on the vascular system, it appears that AQUI-S and MS222 at least, have little effect on catecholamine-mediated changes in red blood cell volume and haematocrit. Also, the use of MS222 is associated with hyperglycemia (Ross and Ross, 1984), indirectly inferring that it does not attenuate catecholamine-stimulated glucogenolysis. The ability of the fish to maintain blood oxygenation and glucose availability *in vivo* even in the presence of anaesthesia may off-set some of the less desirable effects of the anaesthetic on the vasculature, at least during relatively short inductions. If blood oxygenation and metabolite excretion can be maintained and the blood moved through the circulation, then vascular resistance changes due to anaesthesia may, overall have a negligible effect on the efficiency of the cardiovascular system.

Prolonged anaesthesia that causes a continual vasodilation may have deleterious consequences. The blood of snapper, anaesthetised with 100ppm MS222, has been shown to contain anaesthetic concentrations of up to 73 ± 1.8mgL^{-1} (Ryan, 1992). In the present study, perfusion of the Chinook salmon tail with 50ppm MS222 elicited significant vasodilation, until the anaesthetic was washed out. This indicates that the blood vessels of fish could be exposed to anaesthetic concentrations close to or possibly exceeding externally applied concentrations. A profound hypotension that reduces blood flow back to the heart may simulate hypovolemia, another strong stimulator of catecholamine release. It is possible that this may elicit a further increase in plasma catecholamines above those already released to deal with hypoxia. Although there is some evidence for a negative feedback system regulating catecholamine release, the combined stress of hypoxia coupled with a sustained hypotension may 'overload' the system and lead to a continual secretion of catecholamines, particularly if high anaesthetic concentrations block the hypertensive actions of catecholamines. Excessive levels of catecholamines are known to have detrimental effects. Following surgery and black box confinement, *Salmo salar* infected with amoebic gill disease, have been observed to develop extreme hypertension to values above 190 cm H_2O, at which point, the fine blood vessels of the gill and eventually the heart itself, literally burst. (M. Forster, pers.comm.). Both disease and surgery are known to significantly elevate plasma catecholamine levels (Wendelaar Bonga 1997; Fabri et al., 1998).

***In vitro* versus *in vivo* responses.**

Although adrenaline elicited a pressor response when applied to hepatic portal vein rings in the presence of 60ppm AQUI-S and 50ppm MS222 *in vitro*, it is possible that *in vivo*, high concentrations of anaesthetic modulate the response to adrenaline. Prolonged anaesthesia with MS222 has been shown to have direct effects on the heart and neural tissues due to concentration of anaesthetic within these tissues to levels far exceeding external concentrations (Peirce and Peirce 1967; Houston, 1971b; Ryan, 1992; Ryan et al., 1993). The concentration of anaesthetic in these tissues leads to neural inhibition of ventilation and direct effects on the myocardium, eventually leading to cardiac failure. It is possible that high levels of anaesthetic in the tissues cause a hypotension greater than any hypertensive effect that can be generated by the actions of catecholamines. The anaesthetic may essentially 'over-ride' or 'block out' catecholamine effects on the smooth muscle. Ryan et al., (1993) found that rainbow trout hearts perfused with 10mgL^{-1} MS222 showed a fall in both maximum cardiac output and power output of approximately 25%.

The same result was also seen in paced ventricle strips. Additionally, the anaesthetic was concentrated in the tissues of the heart up to 3 times higher than the concentration in the perfusate. It is likely the high lipid solubility of MS222 allows it to bind to the sino-atrial, atrial and ventricular nervous tissue, disrupting nerve transmission through the myocardium (Ryan et al., 1993). There is evidence to show that AQUI-S is concentrated in the edible tissues of Perch (*Bidyanus bidyanus*) (Kildea et al., 2004). During exposure to 15ppm AQUI-S, perch accumulated up to 24mgKg^{-1} of the active ingredient, iso-eugenol. However, levels dropped rapidly within 6 hours and were undetectable by 48 hours following exposure.

In reality, it is often difficult to extrapolate what will occur *in vivo* from *in vitro* results. Results from *in vitro* experiments may not take into account seasonal or other differences between separate fish populations, unless studies are carried out over several seasons. Indeed, it is now well known that trout are more sensitive to catecholamines during summer than during winter (Randall and Perry, 1992). Also, *in vitro* experiments isolate a particular organ or part of a particular system that in the absence of other *in vivo* factors may behave differently from observed results *in vivo*. For example, in the present study, the differences observed between 'new' and 'old' fish in response to perfusion of the tail with anaesthetic may be unique to the isolated tail preparation and not occur when other *in vivo* factors are present. Perfused tissues from the splanchnic circulation of rainbow trout have been shown to be less sensitive to catecholamines than *in vitro* vessel rings (Xu and Olson, 1993a). In a study of the effects of hypoxia on rainbow trout vessels *in vitro*, it was seen that during apparently random 2-3 week periods, efferent branchial and coeliacomesenteric arteries of steelhead trout and efferent branchial arteries of rainbow trout were hyper-reactive to hypoxia compared to previous experiments (Smith et al., 2001). This was attributed to possible seasonal effects. While *in vitro* preparations allow tight control over most experimental variables, interactive effects of different *in vivo* control systems may be lost (Xu and Olson, 1993b). An example of this is the apparent inability of hypoxia to directly stimulate catecholamine secretion from the chromaffin tissue of perfused posterior cardinal vein preparations in salmon or trout (Present study, Perry et al., 2000). However, a series of *in vivo* investigations following on from previous work (Perry et al., 2000) indicated that hypoxia does stimulate catecholamine release from the chromaffin cells, but this response is mediated by internally oriented O_2 chemoreceptors at the gill that can monitor changes in PaO_2 (Reid and Perry, 2003; Perry et al., 2004).

Anaesthetic effects on Haematocrit.

Anaesthesia with MS222 and AQUI-S are associated with an increase in haematocrit (Hct) (Soivio, 1974a; Sovio et al., 1977; Brown, 1993; Hill, 1999; present study). This increase in Hct occurs via a combination of erythrocyte swelling and recruitment from the spleen, both mediated by plasma catecholamines (Randall and Perry, 1992). Resistance to blood flow is due primarily to the viscosity of blood, with velocity being inversely proportional to the total cross-sectional area of a vessel (Satchell 1991). Hct increases viscosity, which is inversely related to shear rate. Therefore, in central veins where shear rate is low due to sub-ambient pressures, blood viscosity increases (Satchell, 1991; Wells and Weber, 1991). However, anaesthesia may alter the dependence of viscosity on shear rate. Following anaesthesia with MS222, adrenergically swollen erythrocytes from rainbow trout did not show a significant increase viscosity at low shear rates (Wells and Weber, 1991). Further to this, Sorensen and Weber (1995) found that red blood cell swelling in the presence of adrenaline reduces the Hct dependence of viscosity in deoxygenated rainbow trout blood, at low shear rates Low viscosity is likely advantageous for flow through the venous circulation where shear rates are also low (Wells and Weber, 1991). Therefore, theoretically, anaesthetic induced vasodilation of peripheral vessels will reduce the velocity of blood while at the same time, viscosity decreases due to adrenergic cell swelling. The increased residence time of the blood cells in the vessels may enhance oxygen delivery to the tissues. The decrease in Hct dependence on viscosity may also help reduce viscosity and therefore load on the heart, in fish where erythrocytes have been sequestered from the spleen. Increasing Hct in this way, by increasing total number of red blood cells would tend to increase viscosity. Additionally, anaesthetic induced vasodilation may act to offset the effects on increased Hct.

Conclusions.

Anaesthesia presents unique physiological challenges to both mammalian and non-mammalian vertebrates due to its direct and modulating influence on cardiovascular homeostasis. There is no natural selection pressure that would specifically adapt an animal to cope with general or local anaesthesia. Therefore animals experiencing general anaesthesia will respond physiologically with mechanisms that have evolved to deal with side effects of anaesthesia. The most potent side effect being hypoxia due to depression of the medullary centres of the brain where ventilation is controlled. Many teleost species are

extremely well adapted to cope with hypoxia. The low capacitance of oxygen in water has selected for this during their evolution and many fish will experience daily bouts of hypoxia due to temperature increases within their environment e.g fish in inter-tidal rock pools or shallow lakes. Therefore, several species have developed specific, well co-ordinated responses to hypoxia that aim to reduce energy demands, maintain blood and tissue oxygenation and enhance glucose availability. During extreme hypoxia, these actions are regulated by the hormones adrenaline and noradrenaline (catecholamines). Catecholamines act to increase systemic blood pressure, enhance haemoglobin-oxygen binding affinity of the red blood cells and thereby CO_2 excretion, increase plasma glucose levels and many other actions that reduce the hypercapnic and acidotic effects caused by hypoxia.

Anaesthetic action varies depending on the type, duration and molecular dynamics of the anaesthetic used. For example, MS222 is used as a local anaesthetic in mammals but induces general anaesthesia in fish. Fish recover rapidly from short inductions with MS222 but mortality rates are high for most species studied when induction is prolonged. Results from the present study indicate that anaesthesia with AQUI-S only stimulates increases in plasma catecholamines during prolonged anaesthesia, which would represent a period of intense hypoxia. This is in agreement with previous studies indicating that an increase in circulating catecholamines is only seen during extreme stress. A short induction with AQUI-S resulted in changes in Hct and MCHC consistent with hypoxia induced red blood cell swelling. However, instead of the hypoxic increase in blood pressure and well-documented bradycardia, salmon showed a fall in dorsal aortic pressure coupled with a significant increase in heart rate. This could be due to a combination of factors such as vagal release of the heart, direct anaesthetic effects on the vascular smooth muscle and possibly a functional baroreflex. Both variables recovered to resting values following anaesthetic withdrawal. In both snapper and salmon, there was a rapid increase in blood pressure following deep anaesthesia. This implies that blood pressure had fallen dramatically during induction, inspite of the extremely high concentrations of plasma catecholamines present in the blood of both species. As an aside, the ability of both species to rapidly clear these high concentrations of catecholamines from the circulation is impressive and indicative of the fine control systems that are in place for these hormones. The entire body seems able to sequester these hormones and attenuate their cardiovascular effects rapidly once normoxia begins. Taking into account the *in vitro* and *in situ* data on blood vessels, it is possible that high concentrations of anaesthetics, acting directly on the vascular smooth muscle inhibit the normal pressor actions of adrenaline during hypoxia.

In situ perfusion of the chromaffin tissue with hypoxic saline failed to elicit catecholamine release, indicating that a local hypoxaemia is not the proximate trigger for catecholamine release in fish. It now appears that O_2 chemoreceptors on the gill lamellae mediate catecholamine release from the chromaffin tissue, although the exact signalling mechanisms underlying this mediation are still unknown.

It appears that the greatest effect of the anaesthetics used in this study was on the smooth muscle of the vascular system where they counteracted the normal pressor response to plasma catecholamines, even in the presence of very high catecholamine concentrations. Effects on catecholamine mediated changes in blood variables such as haematocrit and mean cell haemoglobin concentration appeared to be minimal, if indeed present. The effects of anaesthetics on the vascular system may actually enhance perfusion of the white muscle, which is recruited for high speed burst swimming, although what advantage this would have in a restrained unconscious fish is unclear. It is possible that a hypotensive effect of anaesthetics reduces the workload on the heart due to an increased haematocrit, by reducing vascular resistance. This may also enhance the effect of a reduction of haematocrit dependence on viscosity that has been documented in deoxygenated trout blood.

In conclusion, the effects of circulating plasma catecholamines during anaesthesia with AQUI-S or MS222 seem to be very similar to the effects of catecholamines during severe hypoxia. However, anaesthetic effects on the vascular smooth muscle may counteract or inhibit the normal adrenergic pressor response, although this hypotensive effect may be dependant on certain other unknown hormonal or environmental factors *in vivo*. Fish, like any other animal species, show considerable inter and intra specific variations in the response to various drugs or external agents. The response to anaesthesia in any one fish will depend on factors such as age, weight, health and prior history (e.g has it been anaesthetised previously). The response from a particular species of fish will depend heavily on their adaptive mechanisms to cope with hypoxia. The catecholamine stress response is adaptive for hypoxia and exerts these adaptive effects during anaesthesia. Light anaesthesia may modulate these effects to a degree but it is during prolonged anaesthesia, where anaesthetic concentrations are often concentrated in various tissues that anaesthetics are most likely to inhibit the actions of circulating catecholamines, to the detriment of the fish.

Acknowledgements.

First and foremost I would like to thank my supervisor, Associate Professor Malcolm Forster. His enthusiasm for physiology is legendary in the ranks of both undergraduates and postgraduates alike. Not only is he an excellent teacher, but he is an excellent friend, who supports all his students, no matter what academic level they are at. Over the past 5 years, I have to say it has been great fun working alongside Malcolm and I have (hopefully!) learnt a lot. Without his support and encouragement this project would certainly never have happened. Thank you Malcolm.

My thanks also go to my co-supervisor Associate Professor Bill Davison. Although he didn't play such a direct role in my project work as Malcolm, his straightforward teaching style in undergraduate papers made physiology fun and interesting, rather than confusing and complicated.

Thanks must also go to Lady Diana Issac, Mike Field-Dodgson and the team at Issacs salmon farm. Without the generous support of Lady Issac, much of our work could not have been done easily, particularly when the University Aquarium was being refurbished. Thank you. To Mike, heart felt thanks to you and your team for all the help you have given over the years, especially when I would call you up at short notice and beg for fish. Thanks also for not drowning Malcolm when he came out to help crowd and catch the fish ☺.

My thanks to Alistair Jerret , Suzy Black and Crop and Food Research New Zealand for funding this project and supplying us with AQUI-S. Also thank you for the use of their facilities and fish during our work with the Snapper.

To Gavin Robinson, our physiology technician. Without your help and technical expertise this project would have been impossible. How you manage to co-ordinate stage 2 and 3 labs, honours students, masters students, and Ph.D students is beyond me. All the postgraduates who work with you realise what a fantastic job you are doing and I don't think you know how much we appreciate it. Thank you for everything Gavin, I promise I'll bring in some biscuits for you tomorrow ☺.

To all the other Zoology (SBS) technicians who have helped me over the years, Margaret, Nick, Rosemary, Jo, Franz, John and even though they aren't in the department anymore, Dave Greenwood and Bruce Lindgard Thanks guys.

Big thanks to Lynn and Tracey, our Zoology departmental secretaries. I always intended to only pop down for a quick cuppa, but somehow it always seemed to take about an hour (and somehow I was always the one who was accused of stirring!). Thanks also for helping me sort out little mini-crises like pay etc. What would we post-grads do without you?

Thanks heaps to my office mates over the years. John, with whom I had in depth analytical conversations about Metal Gear Solid and who always let 1 or 2 Wetas escape, just to see if he could make me scream. Cara, who is just starting to get into this monster we call optimistically call 'writing up' and joined us from the real world where they pay you and stuff. Thanks for the hours of conversations we have had and for listening to me ranting on about Word and Prism and other such things. Thanks also for all the times we had a really good laugh and for the indoor rock climbing experience, where I remembered I was afraid of heights! ☺ Good Luck! Jed, thanks for keeping me amused with all your stories about huntin', shootin', fishin' and 4WDin'.

To Duncan's Mum and Dad, Julie and Geoff, thanks for all the games of 500 and Mah jong, I look forward to plenty more. Thanks especially to Julie for all the gins you poured me and to Geoff for all the great food. Thanks for the support guys, xx.

To Tabatha, Sophie and Alex, who always wanted to help me in their own special way, even if it was by walking on the keyboard and deleting what I had just written. Thank you for leaving fur all over my computer, I'll fill up your biscuit bowl in a minute.

Thank you to Sam, Ken and Zak, Jason, Janese, Keryn, Charlie, Leanne and Anneke for keeping me sane and providing me with some of the funniest moments of my life. All the best to you guys.

To Henriette our flying German, thank you for all the hours of conversation and gossip we shared when I felt that work was the last thing I wanted to do. Amazing how our 10-minute coffee breaks turned into 2-hour lunches☺. Thanks for being a great friend. Danke.

To Amanda, my best friend going on 20 years. What can I say Blah, but...Blah. Always great for a laugh (usually at the expense of the opposite sex) and a shopping expedition you can pretty much qualify as my honorary sister. Cheers Manzz!!

Finally, but by no means least, a huge thank you to Duncan. You have been such a huge help in so many ways over the past year and a bit. Thank you for all the cups of tea, games of table tennis, mini-golf, bowling and tennis. Thank you for stopping me from smashing my computer when Word had a fit and decided it didn't like my files. Thank you for driving me nuts sometimes. ☺ Thank you for everything. xxx☺

References.

Abele, B., Hathaway, C.B., Nibbio, B. and Epple, A. (1998). Electrostimulation of Catecholamine release in the eel: Modulation by antagonists and autocrine agonists. *General and Comparative Endocrinology* **109**, 366 - 374.

Aitkenhead, A.R. and Smith, G. (1996). Textbook of anaesthesia,, pp. 773. New York: Churchill Livingstone Inc.

Alpharma Pharmaceuticals. http://alpharmanimalhealth.co.uk. December 2003.

Anderson, G., Mckinley, S.R. and Colavecchia, M. (1997). The use of Clove oil as an anaesthetic for rainbow trout and its effects on swimming performance. *North American Journal of Fisheries Management* **17**, 301-307.

Angel, L. (1980). Effects of anaesthetics on nervous pathways. In *General anaesthesia,* vol. 1 (eds T C Gray ,J F Nunn and J E Utting), pp. 117-139. London: Butterworths.

Aota, S., Holmgren, K.D., Gallaugher, P.E. and Randall, D.J. (1990). A possible role for catecholamines in the ventilatory responses associated with internal acidosis or external hypoxia in the rainbow trout, *Oncorhynchus mykiss. Journal of Experimental Biology* **151**, 57 - 70.

Aota, S. and Randall, D.J. (1993). The effect of exogenous catecholamines on the ventilatory and cardiac responses of normoxic and hyperoxic rainbow trout, *Oncorhynchus mykiss. Journal of Comparative Physiology B* **163**, 138-146.

AQUI-S New Zealand Ltd. www.AQUI-S.com. Accessed December 2003.

Ask, J.A., Stene-Larsen, G. and Helle, K.B. (1980). Atrial á$_2$-adrenoceptors in the trout. *Journal of Comparative Physiology B* **139**, 109-115.

Axelsson, M. (1988). The importance of nervous and humoral mechanisms in the control of cardiac performance in the atlantic cod *Gadus morhua* at rest and during non-exhaustive exercise. *Journal of Experimental Biology* **137**, 287-303.

Axelsson, M., Davison, W., Forster, M.E. and Nilsson, G.E. (1994). Blood pressure control in the Antarctic fish *Pagothenia borchgrevinki. Journal of Experimental Biology* **190**, 265 - 279.

Baum, T. (1977). Fundamental principles governing the regulation of circulatory function. In *Cardiovascular Pharmacology,* (ed. M Antonaccio), pp. 1-43. New York: Raven Press.

Bell, G.R. (1987). An outline of anaesthetics and anaesthesia for salmonids, a guide for fish culturists in British Columbia.

Benoit, E. (1995). Effects of intravenous anaesthetics on nerve axons. *European Journal of Anaesthesiology* **12**, 59-70.

Berenbrink, M., Volkel, S., Heisler, N. and Nikinmaa, M. (2000). O_2 dependant K^+ fluxes in trout red blood cells: the nature of O_2 sensing revealed by the O_2 affinity, cooperativity and pH dependence of transport. *Journal of Physiology* **526.1**, 69 - 80.

Bernier, N.J. and Perry, S.F. (1996). Control of catecholamine and serotonin release from the chromaffin tissue of the atlantic hagfish. *Journal of Experimental Biology* **199**, 2484-2497.

Bernier, N.J. and Randall, D.J. (1998). Carbon dioxide anaesthesia in rainbow trout: effects of hypercapnic level and stress on induction and recovery from anaesthetic treatment. *Journal of Fish Biology* **52**, 621-637.

Bernier, N.J., Mckendry, J.E. and Perry, S.F. (1999a). Blood pressure regulation during hypotension in two teleost species: differential involvement of the renin-angiotesin and adrenergic systems. *Journal of Experimental Biology* **202**, 1677-1690.

Bernier, N.J. and Perry, S.F. (1999b). Cardiovascular effects of angiotensin-I I-mediated adrenaline release in rainbow trout *Oncorhynchus mykiss*. *Journal of Experimental Zoology* **202**, 55-66.

Bernier, N.J., Kaiya, H., Takei, Y. and Perry, S.F. (1999c). Mediation of humoral catecholamine secretion by the renin-angiotesin system in hypotensive rainbow trout (*Oncorhynchus mykiss*). *Journal of Endocrinology* **160**, 351-363.

Bianchini, L. and Pouyssegur, J. (1994). Molecular structure and regulation of vertebrate Na^+/H^+ exchangers. *The Journal of Experimental Biology* **196**, 337-345.

Black, G.W. (1980). Cardiovascular, respiratory and hepatic effects of inhalational anaesthetics. In *General anaesthesia*, vol. 1 eds T C Gray, J F Nunn and J E Utting), pp. 159-187. London: Butterworths.

Boutilier, R.G., Iwama, G.K. and Randall, D.J. (1986). The promotion of catecholamine release in rainbow trout, *Salmo gairdneri*, by acute acidosis: Interactions between red cell pH and haemoglobin oxygen carrying capacity. *Journal of Experimental Biology* **123**, 145 - 157.

Brauner, C.J. and Randall, D.J. (1996a). The interaction between oxygen and carbon dioxide movements in fishes. *Comparative Biochemistry and Physiology* **113A**, 83 - 90.

Brauner, C.J., Gilmour, K.M. and Perry, S.F. (1996b). Effect of haemoglobin oxygenation on Bohr proton release and CO_2 excretion in the rainbow trout. *Respiration Physiology* **106**, 65 - 70.

Brown, L.A. (1993). Anaesthesia and restraint. In *Fish medicine*, (ed. M K Stoskopf), pp. 79-90. Philadelphia: W.B. Saunders Company, Harcourt Brace Jovanovich, Inc.

Burleson, M.L. and Milsom, W.K. (1993). Sensory receptors in the first gill arch of the rainbow trout. *Respiration Physiology* **93**, 97-110.

Burka, J.F., Hammel, K.L., Horsberg, T.E., Johnson, G.R., Rainnie, D.J. and Speares, D.J. (1997). Drugs in Salmonid Aquaculture - A review. *Journal of Veterinary Pharmacology and Theraputics* **20**, 333-349.

Busacker, G.P. and Chavin, W. (1977). Uptake, distribution and turnover of catecholamine radiolabel in the goldfish, *Carassius auratus* L.[1]. *Canadian Journal of Zoology* **55**, 1656-1670.

Bushnell, P.G., Jones, D.R. and Farrell, A.P. (1992). The Arterial system. In *Fish Physiology Vol. XII Part A :The Cardiovascular System*, (eds W S Hoar ,D J Randall and A P Farrell): Academic Press Inc. San Diego.

Canfield, P.J., Quartararo, N., Griffin, D.L., Tsoukalas, G.N. and Cocaro, S.E. (1994). Haematological and biochemical reference values for captive Australian snapper, *Pagrus auratus*. *Journal of Fish Biology* **44**, 849-856.

Charlesworth, P., Jacobson, I., Pocock, G. and Richards, C.D. (1992). The mechanism by which procaine inhibits catecholamine secretion from bovine chromaffin cells. *British Journal of Pharmacology* **106**, 802-812.

Charlesworth, P. and Richards, C.D. (1995). Anaesthetic modulation of nicotinic ion channel kinetics in bovine chromaffin cells. *British Journal of Pharmacology* **114**, 909-917.

Claibourne, J.B. (1998). Acid-base regulation. In *The Physiology of Fishes*, (ed. D H Evans), pp. 177-198. Baton Rouge: CRC Press.

Colletti, A.E. and Olson, K.R. (1988). Catecholamine metabolism by the perfused rainbow trout gill. *The Journal of Experimental Zoology* **248**, 177-184.

Conklin, D.J. and Olson, K.R. (1994). Compliance and smooth muscle reactivity of rainbow trout (*Oncorhynchus mykiss*) vessels *in vitro*. *Journal of Comparative Physiology B* **163**, 657-663.

Conklin, D.J., Chavas, A., Duff, D.W., Weaver (Jnr.), L., Zhang, Y. and Olson, K.R. (1997). Cardiovascular effects of arginine vasotocin in the rainbow trout *Oncorhynchus mykiss*. *Journal of Experimental Biology* **200**, 2821-2832.

Crossland, J. (1981). The Biology of the New Zealand Snapper. Fisheries Research Division (MAF), Occasional publication N^0. 23, pp15.

Damiani, C.E.N., Rossoni, L.V. and Vassallo, D.V. (2003). Vasorelaxant effects of eugenol on rat thoracic aorta. *Vascular Pharmacology* **40**, 59-66.

Davidson, G.W., Davie, P.S., Young, G. and Fowler, R.T. (2000). Physiological responses of rainbow trout (*Oncorhynchus mykiss*) to crowding anaesthesia with AQUI-S. *Journal of the World Aquaculture Society* **31**, 105-114.

Davie, P.S. (1981). Vascular resistance responses of an eel tail preparation:alpha constriction and beta dilation. *Journal of Experimental Biology* **90**, 65-84.

Davis, M.J. and Hill, M.A. (1999). Signalling mechanisms underlying the vascular myogenic response. *Physiological reviews* **79**, 387-423.

Edwards, D., Hall, T., R. and Brown, J.A. (1986). The characteristics and distribution of monamine oxidase (MAO) activity in different tissues of the rainbow trout, *Salmo gairdneri*. *Comparative Biochemistry and Physiology* **84C**, 73-77.

Esler, M., Jennings, G., Lambert, G., Meredith, I., Horne, M. and Eisenhofer, G. (1990). Overflow of catecholamine neurotransmitters to the circulation: Source, fate, and functions. *Physiological reviews* **79**, 963-985.

Fabbri, E., Capuzzo, A. and Moon, T.W. (1998). The role of circulating catecholamines in the regulation of fish metabolism: An overview. *Comparative Biochemistry and Physiology Part C* **120**, 177-192.

Farrell, A.P. (1981). Cardiovascular changes in the lingcod (*Ophiodon elongatus*) following adrenergic and cholinergic drug infusions. *Journal of Experimental Biology* **91**, 293-305.

Farrell, A.P. and Graham, M.S. (1986). Effects of adrenergic drugs on the coronary circulation of the Atlantic salmon (*Salmo salar*). *Canadian Journal of Zoology* **64**, 481-484.

Farrell, A.P. and Jones, D.R. (1992). The Heart. In *Fish Physiology Vol. XII Part A: The Cardiovascular System*, (eds W S Hoar ,D J Randall and A P Farrell): Academic Press Inc. San Diego.

Fievet, B. and Motais, R. (1991). Chapter 3: Na+/H+ exchanges and red blood cell functions in fish. *Advances in Comparative and Environmental physiology* **8**, 79 - 104.

Fiévet, B., Caroff, J. and Motais, R. (1990). Catecholamine release controlled by blood oxygen tension during deep hypoxia in trout: effect on red blood cell Na/H exchanger activity. *Respiration Physiology* **79**, 81-90.

Forster, M.E., Davison, W., Axelsson, M. and Farrell, A.P. (1992). Cardiovascular responses to hypoxia in the hagfish, *Eptatretus cirrhatus*. *Respiration Physiology* **88**, 373 - 386.

Franks, N.P. and Lieb, W.R. (1982). Molecular mechanisms of general anaesthesia. *Nature* **300**, 487-493.

Franks, N.P. and Lieb, W.R. (1994). Molecular and cellular mechanisms of general anaesthesia. *Nature* **367**, 607-614.

Frazier, D.T. and Narahashi, T. (1975). Tricaine(MS222): Effects in ionic conductances of squid axon membranes. *European Journal of Pharmacology* **33**, 313-317.

Fredricks, K.T., Gingerich, W.H. and Fater, D.C. (1993). Comparative cardiovascular effects of four fishery anaesthetics in spinally transected rainbow trout, *Oncorhynchus mykiss*. *Comparative Biochemistry and Physiology* **104C**, 477-483.

Fritsche, R. and Nilsson, S. (1989). Cardiovascular responses to hypoxia in the Atlantic cod, *Gadus morhua*. *Experimental Biology* **48**, 153-160.

Fritsche, R. (1990a). Effects of hypoxia on blood pressure and heart rate in three marine teleosts. *Fish Physiology and Biochemistry* **8**, 85 - 92.

Fritsche, R. and Nilsson, S. (1990b). Autonomic nervous control of blood pressure and heart rate during hypoxia in the cod, *Gadus morhua*. *Journal of Comparative Physiology B* **160**, 293 - 298.

Fromm, P.O., Richards, B.D. and Hunter, R.C. (1971). Effects of some insecticides and MS-222 on isolated perfused gills of trout. *The Progressive Fish-culturist* **33**, 138-140.

Gamperl, A.K., Pinder, A.W., Grant, R.R. and Boutilier, R.G. (1994a). Influence of hypoxia and adrenaline administration on coronary blood flow and cardiac performance in seawater Rainbow trout (*Oncorhynchus mykiss*). *Journal of Experimental Biology* **193**, 209-232.

Gamperl, A.K., Vijayan, M.M. and Boutilier, R.G. (1994b). Epinephrine, norepinephrine and cortisol concentrations in cannulated seawater-acclimated rainbow trout (*Oncorhynchus mykiss*) following black-box confinement and epinephrine injection. *Journal of Fish Biology* **45**, 313-324.

Gantenbein, M., Attolini, L. and Bruguerolle, B. (1996). Potassium channel agonists modify the local anaesthetic activity of bupivacaine in mice. *Canadian Journal of Anaesthesia* **43**, 871-876.

GESAMP. (1997). Towards safe and effective use of chemicals in coastal aquaculture.,, pp. 40: IMO/FAO/UNESCO-IOC/WMO/WHO/IAEA/UN/UNEP Joint group of Experts on the Scientific Aspects of Marine Environmental Protection. Reports and Studies. GESAMP (65).

Gibson, J.S., Cossins, A.R. and Ellory, J.C. (2000). Oxygen-sensitive membrane transporters in vertebrate red blood cells. *Journal of Experimental Biology* **203**, 1395-1407.

Gilderhus, P.A. and Marking, L.L. (1987). Comparative efficacy of 16 anaesthetic chemicals on rainbow trout. *North American Journal of Fisheries Management* 7, 288-292.

Gilmour, K.M., Didyk, N.E., Reid, S.G. and Perry, S.F. (1994). Down regulation of red blood cell B-adrenoreceptors in response to chronic elevation of plasma catecholamine levels in the rainbow trout.

Gilmour, K.M. (1998). Gas Exchange. In *The Physiology of Fishes*. (ed. D H Evans) CRC Press LLC.

Guizouarn, H., Borgese, F., Pellisser, B., Garcia-Romeu, F. and Motais, R. (1993). Role of protein phosphorylation and dephosphorylation in activation and desensitization of the cAMP-dependent $Na^+/H+$ antiport. *The Journal of Biological Chemistry* **268 No. 12**, 8632 - 8639.

Guizouarn, H., Gabillat, N., Motais, R. and Borgese, F. (2001). Multiple transport functions of a red blood cell anion exchanger, tAE1: its role in cell volume regulation. *Journal of Physiology* **535.2**, 497-506.

Healey, E.G. (1964). Anaesthesia of fishes. In *Small animal anaesthesia*, (ed. O Graham-Jones), pp. 59-70. New York: Pergamon Press.

Hill, C.E., Phillips, J.K. and Sandow, S.L. (2001). Heterogeneous control of blood flow amongst different vascular beds. *Medical Research Reviews* **21**, 1-60.

Hill, J.V. (1999). Cardiovascular and respiratory effects of three fish anaesthetics. Ph.D Thesis In *Zoology*,, pp. 113. Christchurch: University of Canterbury, New Zealand.

Hill, J.V. and Forster, M.E. (2004). Cardiovascular responses of Chinook salmon (*Oncorhynchus tshawytscha*) during rapid anaesthetic induction and recovery. *Comparative Biochemistry and Physiology Part C-Pharmacology Toxicology & Endocrinology* **137**, 167-177.

Holeton, G.F. and Randall, D.J. (1967). Changes in blood pressure in the rainbow trout during hypoxia. *Journal of Experimental Biology* **46**, 297 - 305.

Holk, K. and Lykkeboe, G. (1995). Catecholamine-induced changes in oxygen affinity of carp and trout blood. *Respiration Physiology* **100**, 55-62.

Holmgren, S. and Nilsson, S. (1974). Drug effects on isolated artery strips from two teleosts *Gadus morhua* and *Salmo gairdneri*. *Acta physiol. scand.* **90**, 431-437.

Holmgren, S. (1977). Regulation of the heart of a teleost, *Gadus morhua*, by autonomic nerves and circulating catecholamines. *Acta Physiologica Scandinavica* **99**, 62-74.

Houston, A.H., Madden, J.A., Woods, R.J. and Miles, H.M. (1971a). Some physiological effects of handling and tricaine methanesulphonate anaesthetization upon the brook trout, *Salvelinus fontinalis*. *Journal Fisheries Research Board of Canada* **28**, 625-633.

Houston, A.H., Madden, J.A., Woods, R.J. and Miles, H.M. (1971b). Variations in the blood and tissue chemistry of brook trout, *Salvelinus fontinalis*, subsequent to handling, anaesthesia, and surgery. *Journal Fisheries Research Board of Canada* **28**, 635-642.

Hunn, J.B. (1970). Dynamics of MS-222 in the blood and brain of freshwater fishes during anaesthesia. *Investigations in Fish Control* **42**, 3-8.

Hunn, J.B. and Allen, J.L. (1975). Residue dynamics of quinaldine and TFM in rainbow trout. *General Pharmacology* **6**, 15-18.

Imagawa, T., Kitagawa, H. and Uehara, M. (1996). The innervation of the chromaffin cell in the head kidney of the carp, *Cyprinus carpio*; regional differences of the connections between nerve ending and chromaffin cells. *Journal of Anatomy* **188**, 149 - 156.

Ingvast-Larsson, J.C., Axen, V.C. and Kiessling, A.K. (2003). Effects of isoeugenol on in vitro neuromuscular blockade of rat phrenic nerve-diaphragm preparations. *American Journal of Vetinary Research* **64**, 690-693.

Iversen, M., Finstad, B., Mckinley, R.S. and Eliassen, R.A. (2003). The efficacy of metomidate, clove oil, Aqui-S and Benzoak as anaesthetics in Atlantic salmon (*Salmo salar* L.) smolts, and their potential stress-reducing capacity. *Aquaculture* **221**, 549-566.

Iwama, G.K., Mcgeer, J.C. and Pawluk, M.P. (1989). The effects of five fish anaesthetics on acid-base balance, haematocrit, blood gases, cortisol, and adrenaline in rainbow trout. *Canadian Journal of Zoology* **67**, 2065-2073.

Iwama, G.K. and Ackerman, P.A. (1994). Anaesthetics. In *Biochemistry and molecular biology of fishes*, vol. 3 (eds P W Hochachka and T P Mommsen), pp. 1-15. Amsterdam: Elsevier.

Jensen, F.B. (1986). Pronounced influence of Hb-O_2 saturation on red cell pH in tench blood in vivo and in vitro. *Journal of Experimental Zoology* **238**, 119-124.

Jensen, F.B. and Brahm, J. (1995). Kinetics of chloride transport across fish red blood cell membranes. *Journal of Experimental Biology* **198**, 2237-2244.

Jensen, F.B., Fago, A. and E., W.R. (1998). Haemoglobin Structure and Function. In *Fish Respiration*, vol. 17 (eds S F Perry and B Tufts), pp. 356: Academic Press.

Julio, A.E., Monpetit, C.J. and Perry, S.F. (1998). Does acid-base status modulate catecholamine secretion in the rainbow trout (*Oncorhynchus mykiss*)? *The Journal of Experimental Biology* **201**, 3085-3095.

Keene, J.L., Noakes, D.L.G., Moccia, R.D. and Soto, C.G. (1998). The efficacy of clove oil as an anaesthetic for rainbow trout, *Oncorhynchus mykiss* Walbaum. *Aquaculture Research* **29**, 89-101.

Kildea, M.A., Allan, G.L. and Kearney, R.E. (2004). Accumulation and clearance of the anaesthetics clove oil and Aqui-S from the edible tissue of silver perch (*Bidyanus bidyanus*). *Aquaculture* **232**, 265-277.

Koldkjaer Knudsen, P. and Jensen, F.B. (1998). Effects of exhausting exercise and catechoalmines on K+ balance, acid-base status and blood respiratory properties in carp. *Comparative Biochemistry and Physiology Part A* **119**, 301-307.

Laidley, C.W. and Leatherland, J.F. (1988). Cohort sampling, anaesthesia and stocking-density effects on plasma cortisol, thyroid hormone, metabolite and ion levels in rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* **33**, 73-88.

Lapner, K., Monpetit, C.J. and Perry, S.F. (2000). Desensitisation of the chromaffin cell nicotinic receptor does not impede catecholamine secretion during acute hypoxia in Rainbow trout (*Oncorhynchus mykiss*). *The Journal of Experimental Biology* **203**, 1589 - 1597.

Lapner, K. and Perry, S.F. (2001). The role of angoitensin II in regulating catecholamine secretion during hypoxia in rainbow trout *Oncorhynchus mykiss*. *Journal of Experimental Biology* **204**, 4169-4176.

Laurent, P., Holmgren, S. and Nilsson, S. (1983). Nervous and humoral control of the fish heart: structure and function. *Comparative Biochemistry and Physiology* **76A**, 525-542.

Le Bras, Y.M. (1982). Effects of anaesthesia and surgery on levels of adrenaline and noradrenaline in blood plasma of the eel (*Anguilla anguilla* L.). *Comparative Biochemistry and Physiology* **72C**, 141-144.

Lochowitz, R.T., Miles, H.M. and Hafemann, D.R. (1974). Anaesthetic induced variations in the cardiac rate of the teleost, *Salmo gairdneri*. *Comparative and General Pharmacology* **5**, 217-224.

Maffia, M., Rizzello, A., Acierno, R., Rollo, M., Chiloiro, R. and Storelli, C. (2001). Carbonic anhydrase activity in the tissues of the ice-fish *Chionodraco hamatus* and of the red-blooded teleosts *Trematomus bernacchii* and *Anguilla anguilla*. *The Journal of Experimental Biology* **204**, 3983-3992.

Malapert, M., Guizouarn, H., Fievet, B., Jahns, R., Garcia-Romeu, F., Motais, R. and Borgese, F. (1997). Regulation of Na^+/H^+ antiporter in trout red blood cells. *Journal of Experimental Biology* **200**, 353 - 360.

Marking, L.L. and Meyer, F.P. (1985). Are better anaesthetics needed in fisheries? *Fisheries* **10**, 2-5.

Mazeaud, M. and Mazeaud, F. (1973). Excretion and Catabolism of Catecholamines in Fish- Part 1 Excretion Rates. *Comparative and General Pharmacology* **4**, 183-187.

Mazeaud, M. (1974). Monoamine Oxidase and Catechol-O-Methyl Transferase in Tissues of Rainbow Trout (*Salmo gairdneri* Rich., Teleost). *Comparative and General Pharmacology* **5**, 251-253.

Mcfarland, W.N. (1960). The use of anaesthetics for the handling and transport of fishes. *California Fish and Game* **46**, 407-431.

Meechan, J.G. (1990). Does B2 adrenergic agonism affect local anaesthetic efficacy? *Journal of Dentistry* **18**, 254 - 257.

Miller, K.W. (2002). The nature of sites of general anaesthetic action. *British Journal of Anaesthesia* **89**, 17-31.

Mills, C. (1998). Effects of the new anaesthetic, Aqui-S on Stress Responses and Seawater Adaptability of Chinook Salmon (*Oncorhynchus tshawytscha*). BSc. Hons Thesis in *Zoology*, pp. 43. Dunedin: University of Otago, New Zealand.

Mommsen, T.P., Vijayan, M.M. and Moon, T.W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries* **9**, 211-268.

Monpetit, C.J. and Perry, S.F. (1999). Neuronal control of catecholamine secretion from chromaffin cells in the rainbow trout (*Oncorhynchus mykiss*). *Journal of Experimental Biology* **202**, 2059 - 2069.

Monpetit, C.J. and Perry, S.F. (2002). Adrenergic regulation of catecholamine secretion from trout (*Oncorhynchus mykiss*) chromaffin cells. *Journal of Endocrinology* **173**, 187-197.

Montgomery, J.C., Wells, R.M.G. and Parrish, M.J. (1986). Excitory adrenergic innervation in the heart of the marine teleost fish *Girella tricuspidata* (Quoy and Gaimard). *Journal of Experimental Marine Biology and Ecology* **95**, 87-93.

Morales, A.E., Garcia-Rejon, L. and De La Higuera, M. (1990). Influence of handling and/or anaesthesia on stress response in rainbow trout. Effects on liver primary metabolism. *Comparative Biochemistry and Physiology* **95A**, 87-93.

Munday, P.L. and Wilson, S.K. (1997). Comparative efficacy of clove oil and other chemicals in anaesthetization of *Pomacentrus amboinensis*, a coral reef fish. *Journal of Fish Biology* **51**, 931-938.

Nagayama, T., Matsumoto, T., Kuwakubo, F., Fukushima, Y., Yoshida, M., Suzuki-Kusaba, M., Hisa, H., Kimura, T. and Satoh, S. (1999). Role of calcium channels in catecholamine secretion in the rat adrenal gland. *Journal of Physiology* **520.2**, 503-512.

Nekvasil, N.P. and Olson, K.R. (1986). Plasma clearence, metabolism, and tissue accumulation of ^{3}H - labelled catecholamines in trout. *American Journal of Physiology* **250**, R519 - R525.

Nickerson, J.G., Dugan, S.G., Drouin, G., Perry, S.F. and Moon, T.W. (2003). Activity of the unique B-adrenergic Na^{+}/H^{+} exchanger in trout erythrocytes is controlled by a novel B_3-AR subtype. *Am J Physiol Regul Integr Comp Physiol* **285**, R526-R535.

Nikinmaa, M. (1990). Vertebrate Red Blood Cells. eds. Bradshaw, S.D, Burggren, W, Heller, H.C, Ishii, S, Langer, H, Neuweiler, G, Randall, D.J, *Zoophysiology* **28** pp.268 Springer-Verlag.

Nikinmaa, M. and Vihersaari, L. (1993). Pre- and post branchial carbon dioxide content of rainbow trout (*Oncorhynchus mykiss*) blood after catecholamine injection. *Journal of Experimental Biology* **180**, 315-321.

Nikinmaa, M. (1997). Oxygen and carbon dioxide transport in vertebrate erythrocytes: an evolutionary change in the role of membrane transport. *Journal of Experimental Biology* **200**, 369-380.

Nikinmaa, M. and Salama, A. (1998). Oxygen Transport in Fish, In *Fish Physiology* vol. 17, pp. 141 - 184.

Nikinmaa, M. (2001). Haemoglobin function in vertebrates: evolutionary changes in cellular regulation in hypoxia. *Respiration Physiology* **128**, 317-329.

Nilsson, S., Abrahamsson, T. and Grove, D.J. (1976). Sympathetic nervous control of adrenaline release from the head kidney of the cod, *Gadus morhua*. *Comparative Biochemistry and Physiology* **55C**, 123-127.

Nilsson, S. (1994). Evidence for adrenergic nervous control of blood pressure in teleost fish. *Physiological Zoology* **67**, 1347-1359.

NIWA (National Institute of Water and Atmospheric Research). http://www.niwa.cri.nz. Accessed December 2003.

Olsen, R.E. and Ringo, E. (1999). Dominance hierarchy formation in Arctic charr *Salvelinus alpinus* (L.): nutrient digestibility of subordinate and dominant fish. *Aquaculture Research* **30**, 667-671.

Olson, K.R. (1984). Distribution of flow and plasma skimming in isolated perfused gills of three teleosts. *Journal of Experimental Biology* **109**, 97-108.

Olson, K.R. (1991). Vasculature of the fish gill: Anatomical correlates of physiological functions. *Journal of Electron Microscopy Technique* **19**, 389-405.

Olson, K.R., Conklin, D.J., Conlon, M.J., Kellog, M., Smith, M.P., Weaver, L.J., Bushnell, P.G. and Duff, D.W. (1997). Physiological inactivation of vasoactive hormones in rainbow trout. *The Journal of Experimental Zoology* **279**, 254-264.

Olson, K.R. (1998a). Hormone metabolism by the fish gill. *Comparative Biochemistry and Physiology* **119A**, 55-65.

Olson, K.R. (1998b). The Cardiovascular System. In *The Physiology of Fishes*, (ed. D H Evans), pp. 129-154: CRC Press.

Olson, K.R., Russell, M.J. and Forster, M.E. (2001). Hypoxic vasoconstriction of cyclostome systemic vessels: the antecedent of hypoxic pulmonary vasoconstriction? *Am J Physiol Regul Integr Comp Physiol* **280**, R198-R206

Peirce, E.C. and Peirce, E.M. (1967). Effects of tricaine methanesulphonate (MS 222) on the circulation of *Squalus acanthias*. *Bulletin of the Mount Desert Island Biological Laboratory* **7**, 45-47.

Perry, S.F. and Farrell, A.P. (1989). Perfused preparations in comparative physiology. In *Techniques in comparative respiratory physiology - an experimental*

approach, (eds C R Bridges and P J Butler), pp. 223-257. Cambridge: Cambridge University Press.

Perry, S.F., Fritsche, R., Kinkead, R. and Nilsson, S. (1991). Control of catecholamine release *in vivo* and *in situ* in the Atlantic cod (*Gadus morhua*) during hypoxia. *The Journal of Experimental Biology* **155**, 549-566.

Perry, S.F. and Reid, S.G. (1994). The effects of acclimation temperature on the dynamics of catecholamine release during acute hypoxia in the Rainbow trout *Oncorhynchus mykiss*. *The Journal of Experimental Biology* **186**, 289 - 307.

Perry, S.F., Reid, S.G. and Salama, A. (1996). The effects of repeated physical stress on the *B*-adrenergic response of the rainbow trout red blood cell. *Journal of Experimental Biology* **199**, 549 - 562.

Perry, S.F. and Gilmour, K.M. (1996). Consequences of catecholamine release on ventilation and blood oxygen transport during hypoxia and hypercapnia in an elasmobranch (*Squalis acanthias*) and a teleost (*Oncorhynchus mykiss*). *Journal of Experimental Biology* **199**, 2105 - 2118.

Perry, S.F. and Bernier, N.J. (1999). The acute humoral adrenergic stress response in fish: facts and fiction. *Aquaculture* **177**, 285-295.

Perry, S.F., Monpetit, C.J., Julio, A.E. and Moore, K. (1999). The influence of chronic anaemia on catecholamine secretion in the rainbow trout (*Oncorhynchus mykiss*). *Journal of Comparative Physiology B* **169**, 335 - 343.

Perry, S.F., Monpetit, C.J. and Borowska, M. (2000). The effects of acute hypoxia on chemically or neuronally induced catecholamine secretion in Rainbow trout (*Oncorhynchus mykiss*) *in situ* and *in vivo*. *The Journal of Experimental Biology* **203**, 1487 - 1495.

Perry, S.F., Reid, S.D., Gilmour, K.M., Boijink, J.M., Lopes, W.K. and Rantin, F.T. (2004). A comparison of Adrenergic stress responses in three Tropical Teleosts exposed to acute hypoxia. *Am J Physiol Regul Integr Comp Physiol* **(in press)**.

Primmett, D.R.N., Randall, D.J., Mazeaud, M. and Boutilier, R.G. (1986). The role of catecholamines in erythrocyte pH regulation and oxygen transport in rainbow trout (*Salmo gairdneri*) during exercise. *Journal of Experimental Biology* **122**, 139-148.

Prince, A. and Powell, C. (2000). Clove oil as an anaesthetic for invasive field procedures on adult rainbow trout. *North American Journal of Fisheries Management* **20**, 1029-1032.

Quast, U. (1993). Do the K^+ channel openers relax smooth muscle by opening K^+ channels? *Trends in Physiological Sciences* **14**, 332-337.

Quinn, T.P., Olson, A.F. and Konecki, J.T. (1988). Effects of anaesthesia on the chemosensory behaviour of Pacific salmon. *Journal of Fish Biology* **33**, 637-641.

Ragsdale, D.S., Mcphee, J.C., Scheuer, T. and Catterall, W., A. (1994). Molecular determinants of state-dependant block of Na^+ channels by local anaesthetics. *Science* **265**, 1724-1728.

Randall, D.J. (1962). Effect of an anaesthetic on the heart and respiration of teleost fish. *Nature* **195**, 506.

Randall, D. (1982). The control of respiration and circulation in fish during exercise and hypoxia. *Journal of Experimental Biology* **100**, 275-288.

Randall, D.J., Smith, L.S. and Brett, J.R. (1965). Dorsal aortic blood pressures recorded from the rainbow trout (*Salmo gairdneri*). *Canadian Journal of Zoology* **43**, 863-872.

Randall, D.J. and Smith, L.S. (1967). The effect of environmental factors on circulation and respiration in teleost fish. *Hydrobiologia* **29**, 113-124.

Randall, D.J. and Perry, S.F. (1992). Catecholamines. In *Fish Physiology Vol. XII Part B :The Cardiovascular system*, (eds W S Hoar ,D J Randall and A P Farrell), pp. 255-300: Academic Press Inc. San Diego.

Reid, S.D., Lebras, Y. and Perry, S.F. (1993). The in vitro effect of hypoxia on the trout erythrocyte *B*-adrenergic signal transduction system. *Journal of Experimental Biology* **176**, 103 - 116.

Reid, S.D., Furimsky, M. and Perry, S.F. (1994). The effects of repeated physical stress of fasting on catecholamine storage and release in the rainbow trout, *Oncorhynchus mykiss*. *Journal of Fish Biology* **45**, 365-378.

Reid, S.D., Vijayan, M.M. and Perry, S.F. (1996). Modulation of catecholamine storage and release by the pituitary-adrenal axis in the rainbow trout. *Comparative Physiology B* **165**, 665-676.

Reid, S.D., Bernier, N.J. and Perry, S.F. (1998). The adrenergic stress response in fish: control of catecholamine storage and release. *Comparative Biochemistry and Physiology* **Part C**, 1 - 27

Reid, S.F. and Perry, S.F. (1991). The effects and physiological consequences of raised levels of cortisol on Rainbow Trout (*Oncorhynchus mykiss*) erythrocyte beta adrenoreceptors. *Journal of Experimental Biology* **158**, 217 - 240.

Reid, S.G. and Perry, S.F. (2003). Peripheral O_2 chemoreceptors mediate humoral catecholamine secretion from fish chromaffin cells. *Am J Physiol Regul Integr Comp Physiol* **284**, R990-R999.

Ross, L.G. and Ross, B. (1994). Anaesthetic and sedative techniques for fish. Oxford: Blackwell Science.

Ruegg, J.C. (1988). Calcium in Muscle Contraction-2nd edition (1st edition printed as vol. 19 of series Zoophysiology). Berlin: Springer-Verlag.

Ryan, S.N. (1991). Distribution of MS-222 in the tissues of two marine teleosts: *Chrysophrys auratus* and *Pagothenia borchgrevinki*. In *ZoologyDepartment*,, pp. 80. Auckland, New Zealand: University of Auckland.

Ryan, S.N. (1992). The dynamics of MS-222 anaesthesia in a marine teleost (*Pagrus auratus*: Sparidae). *Comparative Biochemistry and Physiology* **101C**, 593-600.

Ryan, S.N., Davie, P.S., Gesser, H. and Wells, R.M.G. (1993). The effect of MS-222 on paced ventricle strips and the perfused heart of rainbow trout, *Oncorhynchus mykiss*. *Comparative Biochemistry and Physiology* **106C**, 549-553.

Salama, A. and Nikinmaa, M. (1989). Species differences in the adrenergic response of fish red blood cell: studies on whitefish, pikeperch, trout and carp. *Fish Physiology and Biochemistry* **6**, 167 - 173.

Satchell, G.H. (1971). Fish Circulation. eds. P.W Brian, G.M Hughes, G Salt, E.N Willmer, *Cambridge monographs in experimental biology* **No.18** London: Cambridge University Press.

Satchell, G.H. (1991). Physiology and form of fish circulation. Cambridge: Cambridge University Press. pp.235

Schoettger, R.A., Walker, C.R., Marking, L.L. and Julin, A.M. (1967). MS-222 as an anaesthetic for Channel Catfish: its toxicity, efficacy and muscle residues. *Investigations in Fish Control* **17**, 3-14.

SeaFIC (New Zealand Seafood Industry Council). http://seafood.co.nz Accessed December 2003.

Sellgren, J., Ponten, J. and Wallin, B.G. (1992). Characteristics of muscle nerve sympathetic activity during general anaesthesia in humans. *Acta Anaesthesiology Scandanavia* **36**, 336-345.

Shipton, T. (1999). How do anaesthetics work? *South African Journal of Anaesthesiology and Analgesia* **June**, 2-4.

Small, B.C. (2003). Anaesthetic efficacy of metomidate and comparison of plasma cortisol responses to tricaine methanesulfonate, quinaldine and clove oil anaesthetized channel catfish *Ictalurus punctatus*. *Aquaculture* **218**, 177-185.

Smit, G.L., Hattingh, J. and Burger, A.P. (1979). Haematological assessment of the effects of the anaesthetic MS 222 in natural and neutralized form in three freshwater fish species: interspecies differences. *Journal of Fish Biology* **15**, 633-643.

Smith, M.P., Russell, M.J., Wincko, J.T. and Olson, K.R. (2001). Effects of hypoxia on isolated vessels and perfused gills of rainbow trout. *Comparative Biochemistry and Physiology Part A* **130**, 171-181.

Soivio, A., Mälkönen, M. and Tuurala, O. (1974). Effects of asphyxia and MS-222 anaesthesia on the circulation of the kidney of *Salmo gairdneri* Richardson. A microscopical study. *Annales Zoologici Fennici* **11**, 271-275.

Soivo, A., Westman, K. and Nyholm, K. (1974). The influence of changes in oxygen tension on the haematocrit value of blood samples from asphyxic rainbow trout (*Salmo gairdneri*). *Aquaculture* **3**, 395-401.

Soivio, A., Nyholm, K. and Huhti, M. (1977). Effects of anaesthesia with MS 222, neutralized MS 222 and benzocaine on the blood constituents of rainbow trout, *Salmo gairdneri*. *Journal of Fish Biology* **10**, 91-101.

Soivio, A. and Hughes, G.M. (1978). Circulatory changes in secondary lamellae of *Salmo gairdneri* gills in hypoxia and anaesthesia. *Annales Zoologici Fennici* **15**, 221-225.

Sorensen, B. and Weber, R.E. (1995). Effects of oxygenation and the stress hormones adrenaline and cortisol on the viscosity of blood from the trout *Oncorhynchus mykiss*. *Journal of Experimental Biology* **198**, 953 - 959.

Soto, C.G. and Burhanuddin. (1995). Clove oil as a fish anaesthetic for measuring length and weight of rabbitfish (*Siganus lineatus*). *Aquaculture* **136**, 149-152.

Stehly, G.H. and Gingerich, W.H. (1999). Evaluation of Aqui-S (efficacy and minimum toxic concentration) as a fish anaesthetic/sedative for public aquaculture in the United States. *Aquaculture Research* **30**, 365-372.

Strange, R.J. and Schreck, C.B. (1978). Anaesthetic and handling stress on survival and cortisol concentration in yearling chinook salmon (*Oncorhynchus tshawytscha*). *Journal Fisheries Research Board of Canada* **35**, 345-349.

Summerfelt, R.C. and Smith, L.C. (1990). Anaesthesia and surgery. In *Methods for Fish Biology*, (eds C B Schreck and P B Moyle). Bethseda: American Fish Society.

Sumpter, J.P., Pottinger, T.G., Rand-Weaver, M. and Campbell, P.M. (1993). The wide-ranging effects of stress on fish. *Perspectives in comparative endocrinology*, 535-538.

Sundin, L. and Nilsson, G.E. (1997). Neurochemical mechanisms behind gill microcirculatory responses to hypoxia in trout: in vivo microscopy study. *American Journal of Physiology* **272**, R576 - R585.

Tabrizchi, R. and Pang, C.C.Y. (1992). Effects of drugs on body venous tone, as reflected by mean circulatory filling pressure. *Cardiovascular Research* **26**, 443-448

Taylor, E.W. (1992). Nervous control of the heart and cardiorespiratory interactions. In *Fish Physiology Vol XII Part B : The Cardiovascular system*, (eds W S Hoar ,D J Randall and A P Farrell), pp. 343-387. San Diego: Academic Press Inc.

Taylor, P.W. and Roberts, S.D. (1999). Clove oil: an alternative anaesthetic for aquaculture. *North American Journal of Aquaculture* **61**, 150-155.

Tetens, V. and Christensen, N.J. (1987). Beta-adrenergic control of blood oxygen affinity in acutely hypoxia exposed rainbow trout. *Journal of Comparative Physiology B* **157**, 667 - 675.

Tetens, V. and Lykkeboe, G. (1988). Potency of adrenaline and noradrenaline for *B*-adrenergic proton extrusion from red cell of rainbow trout, *Salmo gairdneri*. *Journal of Experimental Biology* **134**, 267 - 280.

Tonner, P.H. and Miller, K.W. (1995). Molecular sites of general anaesthetic action on acetylcholine receptors. *European Journal of Anaesthesiology* **12**, 21-30.

Trudell, J.R. and Bertaccini, E. (2002). Molecular modelling of specific and non-specific anaesthetic interactions. *British Journal of Anaesthesia* **89**, 32-40.

Ungell, A.-L. and Nilsson, S. (1979). Metabolic degradation of [H^3]-adrenaline in the atlantic cod *Gadus morhua*. *Comparative Biochemistry and Physiology* **64C**, 137-141.

Ungell, A.-L. (1985). Accumulation of intra-arterially administered [H^3] noradrenaline in various tissues of the atlantic cod, *Gadus morhua*. *Comparative Biochemistry and Physiology* **81C**, 25-28.

Urban, B.W. and Bleckwenn, M. (2002). Concepts and correlations relevant to general anaesthesia. *British Journal of Anaesthesia* **89**, 3-16.

Van Der Boon, J., Van Den Thillart, G.E.E.J.M. and Addink, A.D.F. (1991). The effects of cortisol administration on intermediary metabolism in teleost fish. *Comparative Biochemistry and Physiology* **100A**, 47-53.

Virkki, L.V. and Nikinmaa, M. (1994). Activation and physiological role of Na+/H+ exchange in lamprey (*Lampetra fluviatilis*) erythrocytes. *The Journal of Experimental Biology* **191**, 89-105.

Walker, C.R. and Schoettger, R.A. (1967). Residues of MS-222 in four salmonids following anaesthesia. *Investigations in Fish Control* **15**, 3-11.

Walsh, P.J., Wood, C.M. and Moon, T.W. (1998). Red blood cell metabolism. In *Fish Physiology*, vol. 17, pp. 41 - 59.

Wartenberg, H.C., Wang, J., Rehberg, B., Urban, B.W. and Duch, D.S. (1994). Molecular actions of pentobarbitone on sodium channels in lipid bilayers: role of channel structure. *British Journal of Anaesthesia* **72**, 668-673.

Wells, R.M.G. and Weber, R.E. (1991). Is there an optimal haematocrit for rainbow trout, *Oncorhynchus mykiss* (Walbaum)? An interpretation of recent data based on blood viscosity measurements. *Journal of Fish Biology* **38**, 53 - 65.

Wendelaar Bonga, S.E. (1997). The stress response in fish. *Physiological reviews* 77, 591 - 625.

Whiteley, N.M. and Egginton, S. (1999). Antarctic fishes have a limited capacity for catecholamine synthesis. *Journal of Experimental Biology* **202**.

Wood, C.M. and Shelton, G. (1975). Physical and adrenergic factors affecting systemic vascular resistance in the rainbow trout: a comparison with branchial vascular resistance. *Journal of Experimental Biology* **63**, 505-523.

Wood, C.M. (1976). Pharmacological properties of the Adrenergic Receptors Regulating Systemic Vascular Resistance in the Rainbow trout. *Journal of Comparative Physiology B* **107**, 211-228.

Wood, C.M. and Simmons, H. (1994). The conversion of plasma HCO_3^- to CO_2 by rainbow trout red blood cells *in vitro*: adrenergic inhibition and the influence of oxygenation status. *Fish Physiology and Biochemistry* **12**, 445 - 454.

Woody, C.A., Nelson, J. and Ramstead, K. (2002). Clove oil as an anaesthetic for adult sockeye salmon: field trials. *Journal of Fish Biology* **60**, 340-347.

Xu, H. and Olson, K.R. (1993a). Significance of Circulating Catecholamines in Regulation of Trout Splanchnic Vascular Resistance. *The Journal of Experimental Zoology* **267**, 92-96.

Xu, H.Y. and Olson, K.R. (1993b). Evidence for multiple adrenoceptor sites in rainbow trout vasculature. *Journal of Comparative Physiology B* **163**, 470-476.

Yeh, J.-L., Yang, T.-H., Liang, J.-C., Huang, Y.-C., Lo, Y.-C., Wu, J.-R., Lin, Y.-T. and Chen, I.-J. (2000). Isoeugenodilol: A vasorelaxant alpha/beta-Adrenoceptor Blocker with Antioxidant activity. *Drug Development Research* **51**, 29-42.

Zweieten, P.A. (1988). The role of adrenoreceptors in circulatory and metabolic regulation. *American Heart Journal* **116**, 1384-1392.